Chemical characterization of *Malpighia emarginata* DC. bioresidues and exploration of their colourant potential

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Abbreviations

a*	Red/green chromaticity
AAPH	2,2'-azobis(2-amidinopropan)
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ADD	Allowable Daily Dose
AGS	Gastric carcinoma
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
ATCC	American Type Culture Collection
ATF	Trifluoroacetic Acid
b*	Blue/yellow chromaticity
CaCo	Intestinal carcinoma
CCRD	Central Composite Rotatable Design
CE	Circular Economy
CFU	Colony Forming Unit
CIE	International Lighting Commission
COD	Cyaninidin-*O*-deoxyhexoside
CV	Coefficient of variance
DAD	Diode Array
DMEM	Eagle Medium Modified by Dulbecco
DMSO	Dimethyl Sulfoxide
DMSO	Dimethyl Sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EAAE	Enzyme Assisted Aqueous Extraction
EACP	Enzyme Assisted Cold Pressing
EAE	Enzyme Assisted Extraction
EC_{50}	Extract concentration corresponding to 50% of antioxidant activity
ECACC	European Animal Cell Culture Collection
ESI	Electrospray Ionization
EU	European Union

FAME	Fatty Acid Methyl Esters
FAO	Food and Agriculture Organization
FBS	MCF-7 and NCI-H460
FDA	Food and Drug Administration
FID	Flame ionization detector
FSC	Food Supply Chain
GC-FID	Gas Chromatography-Flame Ionization Detector
GI_{50}	Sample concentration that inhibits 50% of cell growth
HAE	Heat Assisted Extraction
HBSS	Hank's Saline Solution
HDL	High Density Lipoprotein
HPLC	High Performance Chromatography
HPLC-DAD	High Performance Liquid Chromatography with a Diode-array Detector
HPLC-DAD-ESI/MS	Mass Spectrometry using the electrospray ionization interface
HPLC-fluorescence	High Performance Chromatography-Fluorescence
HPLC-RI	High Performance Chromatography-Refractive Index
INS	International Numbering System
INT	Iodonitrotetrazolium
JECFA	Joint Committee of Experts on Food Additives and Contaminants
LDL	Low Density Lipoprotein
MAE	Microwave Assisted Extraction
MBC	Minimum Bactericidal Concentration
MCF-7	Breast carcinoma
MDA	Malodialdehyde
MFC	Minimum Fungicidal Concentration
MHB	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MS	Mass Spectrometer
Mt	Megatone
MUFA	Monounsaturated Fatty Acids
NCI-H460	Lung carcinoma

NCTC	National Collection of Type Cultures
NED	N-(1-naphthyl) ethylenediamine hydrochloride
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate-saline Buffer
PE	Erythrocyte Population
PEF	Pulsed Electric Field Extraction
PLE	Pressurized Liquid Extraction
PLP2	Primary culture of pig liver cells
POD	Pelargonidin-*O*-deoxyhxoside
PUFA	Polyunsaturated Fatty Acids
RSM	Surface Analysis Method
SFA	Saturated Fatty Acids
SFB	Fetal Bovine Serum
SFE	Supercritical Fluid Extraction
SRB	Sulforhododamine B
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic Acid
TSB	Triptych Soy Broth
UAE	Ultrasound Assisted Extraction
UFLC-PDA	Ultra Fast Liquid Chromatography and Photodiode Array Detection
UPLC	Ultra Performance Liquid Chromatography
USA	The United States of America
WFD	European Waste Framework Directive
WHO	World Health Organization

Abstract

From the Industrial Revolution, humanity has been suffering the consequences of a system that only focus on economic growth, neglecting environment quality and, consequently, population's health. Horticulture is one of the industrial sectors where the greatest amount of organic waste is generated. These biowastes are further discarded and devalued. However, these by-products are excellent sources of pigments, phenolic compounds, dietary fibres, sugar derivatives, organic acids, minerals and vitamins. All these compounds have health beneficial properties, so their reuse is an effective and promising alternative for industry and environment. Many authors have studied different vegetable matrices and found molecules of high industrial interest in their composition. Due to its high vitamin C, carotenoids and anthocyanins content, *Malpighia emarginata* DC. (acerola) is known for its nutritional and functional properties.

In this work the nutritional and chemical characterization of *M. emarginata* bioresidues were studied, as well as the evaluation of bioactive potential. Furthermore, a study of extraction optimized process was performed, in order to obtain a natural colourant pigment rich in anthocyanins.

The nutritional profile was determined using official methodologies for the analysis of food products (AOAC) and the results showed the carbohydrates as the predominant macronutrient and low values of fat content. The chemical profile was evaluated determining free sugars by HPLC-RI, organic acids by UFLC-PDA, fatty acids by GC-FID and phenolic compounds (anthocyanin and non-anthocyanin) by HPLC-DAD-ESI/MS. Fructose, glucose and sucrose were identified, highlighting fructose as the main free sugar compound. Also, oxalic, malic, ascorbic and succinic acids were identified, being malic acid the most abundant organic acid. The fatty acids profile revealed the presence of fourteen compounds, being mostly represented by saturated fatty acids, with oleic acid as the majority. The phenolic composition evidenced the presence of six non-anthocyanin compounds and two anthocyanins, standing out the isorhamnetin-*O*-pentosyl-hexoside and cyanidin-*O*-deoxyhexoside as the major compounds, respectively.

The bioactive potential was evaluated throught antioxidant activity by TBARS and OxHLIA methodologies; antimicrobial activity was determined by the microdilution method

using clinical isolated from Gram-positive and Gram-negative bacteria and a fungi strain; cytotoxicity was tested in four human tumour cell lines (MCF-7, NCI-H460, AGS and CaCo) and in a non-tumour primary cell culture (PLP2), using sulforrodamine B assay. Finally, anti-inflammatory activity was determined in RAW264.7 cells.

Based on the obtained values, the presence of antioxidant and anti-inflammatory action was demonstrated. Also, the antimicrobial activity revealed promising results, with good bacteriostatic and fungistatic effects. Concerning cytotoxic evaluation, the extract revealed a greater capacity to inhibit the half tested tumour cell lines than to inhibit non-tumour cells.

Subsequently, the heat assisted extraction (HAE) methodology was used for the extraction optimization process, applying the response surface analysis method (RSM). In this study it was found that the optimal extraction conditions for obtaining an extract rich in anthocyanin compounds were: t = 24.8 min, T = 40.5°C and % ethanol = 11.8. The HAE extraction process showed a relevant yield value of 57.1%, being quantified a total anthocyanins content of 2.54 mg/g extract.

Overall, this study highlights the excellent composition in compounds of interest and bioactive potential of *M. emarginata* fruits, as well as, its potential as an alternative and promising source of anthocyanin compounds.

Keywords: Heat-assisted extraction; anthocyanins; bioresidues; *Malpighia emarginata* DC.

Resumo

Com o evoluir do tempo, a humanidade tem vindo a acompanhar as consequências de um sistema remanescente da Revolução Industrial que, por visar apenas a produtividade com foco no crescimento económico, descurou a qualidade do ambiente e, consequentemente, a saúde da população. A hortofrutícultura é um dos sectores industriais onde se gera maior quantidade de resíduos orgânicos, que são descartados e desvalorizados. Contudo, esses subprodutos são excelentes fontes de pigmentos, compostos fenólicos, fibras alimentares, derivados de açúcar, ácidos orgânicos, minerais e vitaminas que possuem propriedades benéficas à saúde, sendo o seu reaproveitamento uma alternativa eficaz e promissora para a indústria e para o ambiente. São várias a matrizes vegetais que têm vindo a ser estudadas por diversos autores, que constataram a sua interessante composição em moléculas de elevado interesse para o setor industrial. *Malpighia emarginata* DC. (acerola) é um fruto conhecido pelas suas propriedades nutricionais e funcionais, devido ao seu elevado conteúdo de vitamina C, carotenóides e antocianinas.

Neste trabalho foi estudada a caracterização nutricional e química de biorresíduos de *M. emarginata*, bem como a avaliação do seu potencial bioativo. Além disso, foi realizado um estudo do processo de otimização da extração, a fim de se obter um pigmento corante natural rico em antocianinas.

O perfil nutricional foi determinado através de metodologias oficiais para análise de produtos alimentares (AOAC), e os resultados mostraram os hidratos de carbono como o macronutriente predominante e baixo teor de gordura. O perfil químico foi avaliado determinando os açúcares livres por HPLC-RI, os ácidos orgânicos por UFLC-PDA, os ácidos gordos por GC-FID e os compostos fenólicos (antociânicos e não antociânicos) por HPLC-DAD-ESI/MS. Foram identificados frutose, glucose e sacarose, destacando-se a frutose como o principal composto de açúcares livres. Também foram identificados os ácidos oxálico, málico, ascórbico e succínico, sendo o ácido málico o ácido orgânico mais abundante. O perfil de ácidos gordos revelou a presença de catorze compostos, tendo sido na sua maioria representados por ácidos gordos saturados, com o ácido oleico como maioritário. A composição fenólica evidenciou a presença de seis compostos não antociânicos e dois antociânicos, destacando-se a isoramnetina-*O*-pentosil-hexosídeo e a cianidina-*O*-

desoxihexosídeo como compostos maioritários, respectivamente. O potencial bioativo foi avaliado através da atividade antioxidante pelas metodologias TBARS e OxHLIA; a atividade antimicrobiana foi determinada pelo método de microdiluição utilizando isolados clínicos de bactérias Gram-positivos e Gram-negativos e uma estirpe de fungo; a citotoxicidade foi testada em quatro linhas de células tumorais humanas (MCF-7, NCI-H460, AGS e CaCo) e numa cultura de células primárias não tumoral (PLP2), utilizando o ensaio de sulforrodamina B. Finalmente, a atividade antiinflamatória foi determinada em células RAW264.7.

Com base nos valores obtidos, foi demonstrada a presença de ação antioxidante e antiinflamatória. Também a atividade antimicrobiana revelou resultados promissores, com bons efeitos bacteriostáticos e fungistáticos. Relativamente à avaliação citotóxica, o extracto revelou uma maior capacidade de inibir as linhas de células tumorais testadas do que de inibir as células não tumorais.

Posteriormente, a metodologia de extração assistida por calor (HAE) foi utilizada para o processo de otimização da extração, aplicando-se o método de análise de superfície de resposta (RSM). Neste estudo verificou-se que as condições ótimas de extração para a obtenção de um extrato rico em compostos antocianínicos foram: t = 24.8 min, T = 40.5°C e % etanol = 11.8%. O processo de extração de HAE apresentou um valor relevante de rendimento de 57.1%, sendo quantificado um teor total de antocianinas de 2.54 mg/g de extrato.

No geral, este estudo destaca a excelente composição em compostos de interesse e o potencial bioativo de *M. emarginat*a frutas, bem como, o seu potencial como fonte alternativa e promissora de compostos antociânicos.

Palavras-chave: Extração assistida por calor; antocianinas; biorresíduos; *Malpighia emarginata* DC.

Bibliography Review

1. Introduction

1.1 Biowaste from food industry

With the evolution of time, humanity has been following the consequences of a system that comes from the Industrial Revolution that, by focusing only on productivity for the economic growth, neglected the quality of the environment and, consequently, the population's health (Asioli et al., 2017). Thus, food production has enabled man to adopt a lifestyle with intensive needs and desires for natural resources, which leads to a negative environmental impact (Aschemann-Witzel et al., 2019). Ecological disturbances have become more numerous, widespread and more intense, turning a problem on a global scale, being the man and his practices the main threat to the environment (Caldeira et al., 2019). In this sense, the development of the horticultural sector emerges exponentially, as a global need due to the growing demand for food of vegetable origin.

So, the food industry produces a lot and different types of waste, namely industrial effluents (biological and chemical) and solid residues. This is one of the most regulated sectors in Europe (Pap et al., 2014) and that generates a greater amount of biowaste (**Table 1**). Considering data of 2011, the European Union (EU) produced a total of 129.2 Mt (megaton) of residues, most of them coming from the fruit and vegetable sector, with around 28.1 and 313.3 Mt, respectively (Caldeira et al., 2019).

Table 1. Food waste in EU calculated for each food group and food supply chain (FSC) in 2011. (Source: Caldeira, 2019).

INDUSTRY	BY-PRODUCTS	TOTAL (Mt)
Meat	Blood, guts, bones, intestines, skin, fats, hair and feathers	14.2
Fish	Heads, viscera, tails, skin, spines and shells	4.2
Dairy	Milk whey	6.8
Eggs	Shell	1.8
Cereals	Leaves and seeds	15.6
Fruit	Leaves, seeds, skins, stems and pulp	28.1
Vegetables	Leaves, seeds, skins, stems and pulp	31.3
Potatoes	Peel	9.4
Sugar beets	Pulp, molasses and yeast	5.1
Oil crops	Leaves, marc, gum and soaps	12.7

According to the European Waste Framework Directive (WFD), biowaste is defined as *"biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises and comparable waste from food processing*

plants". In its Article 22, the WFD prescribes that Member States shall take measures to encourage: *"a) the separate collection of biowaste with a view to the composting and digestion of biowaste; b) the treatment of biowaste in a way that fulfils a high level of environmental protection; c) the use of environmentally safe materials produced from biowaste".*

Annually, in Europe, food waste is around 1.3 billion tons, of which approximately 700 million tons are from agriculture and are discarded in an inappropriate way for the environment. This leads to a growing concern of the agrofood industry regarding their use. These residues result essentially from the processing of grains, fruits and vegetables, and have a high potential for reuse (Woiciechowski et al., 2013; Toop et al., 2017).

Biological effluents (liquid wastes) result from production processes and must be treated according to their concentration of organic matter. These bioresidues come from washing floors, transport boxes, among others. Otherwise, solid waste results from the raw material and the applied processes. This biowaste includes bagasse, fruits and vegetable peels, expired or out of specification food products, food scraps from restaurants, supermarkets, among others.

All of these categories of residues need a different kind of treatment and specialized professional labour, since they have highly polluting substances that can harm the environment (Caldeira et al., 2019). Thus, the manufacturng sector gets unrecovered financial expenses, resulting in an economic loss for the industries. (Almeida, 2016).

Within the food industry, the fruit and vegetable processing sector represent a huge proportion of the total agrofood industry. **Table 2** shows the percentage of solid waste that is obtained in the production of various foods associated to the fruit and vegetable sector. Artichoke and orange juice, both with a percentage between 60 and 65, evidenced a large amount of bioresidue not consumed and/or not used, followed by pepper (50-60%), chard (48%) and leek (47%). Also, it is shown that the processing of specific vegetables and fruits generates residues consisting of skins, seeds, leaves and roots among, others.

Table 2. Types and percentages of solid waste from fruit and vegetable processing in European Community. (Source: Conesa Dominguez, 2018).

RAW MATERIAL	WASTE TYPE	% OF TOTAL WASTE (per year)
Tomato	Skin, seed, rotten	15
Pepper	Placenta, skin	50-60
Artichoke	Bracts, stems	60-65
Vetch	Extremes	28
Leek	Leaves, roots	47
Garlic	White parts	17
Chard	Dry leaves	48
Spinach	Dry leaves	13
Cantaloupe	Skins, bones	22-28
Plum	Skins, bones	10-25
Orange, tangerine	Skin, peel, seeds	40-45
Orange (juice)	Skin, peel, seeds	60-65
Pear	Skin, petioles, endocarp	42-45

The agrofood by-products, particularly fruits and vegetables, are renewable sources that can originate new added-value ingredients with functional compounds and properties. The processing of natural matrices has been identified as excellent sources of phenolic compounds, presenting bioactivities of interest to the food industry, aiming at their use as functional ingredients (Faustino et al., 2019).

These wastes are mainly primary residues that can be transformed into resources by using intensified conversion processes. Thus, potentially sustainable bioproducts, such as energy, fertilizers, materials and molecules could be produced (Gontard et al., 2018). Therefore, to minimize costs, food industry should focus in avoid waste generation and in their exploitation. Utilization of by-products and wastes as raw materials represent a promising and viable method to the industrial develop (Pap et al., 2014).

In order to minimize the global production cost and its environmental impact, this research proposes the use of a bioresidue from the fruit industry. Its nutritional, chemical and bioactive potential was studied, as well as its colouring pigments for commercial applications.

1.1.1 Valorization and applicability

Associated with high level of vegetable matrices consumption, high waste production is a daily reality. In this sense, foods that do not meet the requirements to be sell, due to their unattractive appearance or because of an advanced stage of maturation, become biowastes.

These products could be used for other purposes, reducing production costs and wastes generation, contributing to its valorization and reduction of the rate of waste (Kazemi et al., 2019).

In recent years, the valorization of residues has gained prominence as an alternative option to waste disposal, such as landfill. This can be referred to as any industrial process that involves the reuse, recycling or composting of wastes, generating useful products or energy (Kabongo, 2013). The Circular Economy (CE) concept propose that waste becomes a raw material in another process, so it is transformed into a new product (Lett, 2014). Waste valorization is central to the concept of CE, which is currently gaining considerable policy relevance. The term of CE is thus a powerful bridging concept to foster the fundamental links among resource use, waste and emissions. It also contributes to integrate environmental (output-related) and economic (input-related) policies (Mayer et al., 2019).

As previously mentioned, the food industry generates huge amounts of wastes that are characterized by their high moisture content, high biological instability and high organic load, which promote microbial activity and, therefore, are difficult to manage. Improper disposal practices for these wastes cause environmental problems such as toxicity to aquatic life, contamination of surface and ground waters, alteration of soil quality, among others. Hence, the global legislation requirements for waste management and disposal have become increasingly restrictive over the past decade (Chandrasekaran et. al, 2012).

Due to the great interest in reducing and reusing organic waste, various techniques have been developed to transform it into useful products, namely by-products. According the European Waste Framework Directive (2008/98/EC), by-product is defined as: "*a substance or object resulting from a production process, the primary aim of which is not the production of that item. By-products are regarded non-waste, if the following conditions are met:*

a) Further use is certain;

b) Can be used directly, without further processing;

c) Produced as an integral part of a production process;

d) Further use is lawful and use will not lead to overall adverse environmental impacts".

The most widespread is the generation of biofuels such as: biogas, biohydrogen, biodiesel. These processes are the simplest and cheapest options. Another way of valorization

is the development of biomaterials (enzymes, biofertilizers, biopolymers) through microbial activity. One of the latest growing is the development of absorbents for some types of waste of biological origin to treat wastewater (Nayak and Bhushan, 2019).

In addition, another application is the extraction and recovery of value-added components (bioactive compounds), used in food, pharmaceutical and cosmetic industries (Nayak and Bhushan, 2019).

In the particular case of the food industry, there have been several studies developed for the transformation of biowaste into bioproducts, for example, by microbiological and enzymatic methods. The presence of large quantity of interesting compounds in these residues is notorious, as stated by the scientific community (Faustino et al., 2019; Torres-León et al., 2021).

The greatest source of bioactive compounds is obtained from residues generated in the processing of fruits and vegetables. Its by-products are excellent sources of pigments, phenolic compounds, dietary fibers, sugar derivatives, organic acids, minerals and others, that demonstrate several beneficial health attributes, such us: antibacterial, antitumor, antiviral, antimutagenic and cardioprotective activities (Dilas et al., 2009).

Actually, the recovery of biological waste has gained prominence as an alternative option to eliminate its accumulation, reduce the cost of its degradation and promote the development of circular economy. From this, the use of waste becomes an important practice to produce new ingredients for food products and to reduce environmental problems. As these residues are generated in large quantities by the fruit and vegetable processing industries, they become a good alternative to obtain extracts rich in bioactive compounds.

Studies performed by Dias et al. (2019) and da Silva Veloso et al. (2020) have shown that fruit residues, such as kiwi and pomegranate, have promising profiles of phenolic compounds and bioactive potentials. These results enabled them to be implemented in different industrial areas, particularly by the food industry.

In the case of *Malpighia emarginata* DC. process, the red residue called “bagasse” has a high content of anthocyanins and ascorbic acid (Moreira, 2007). Thus, its use represents a great commercial potential. As an example, a microencapsulated extract of the *M. emarginata* bagasse has been used as functional ingredient with antioxidant and/or colouring properties in the food industry.

1.2 Additives in the food industry

The civilizations growth leaded to a greater demand of food and of products with best quality. In this way, it was necessary to develop techniques capable of improving the organoleptic characteristics of foods and prolonging their durability (Carocho et al., 2014). Initially, this type of methodologies was used in a natural way. In ancient Egypt, several primary techniques were used to extend food shelf life, since the climate was not suitable for storage. Some practices included adding salt, curing, sun drying and burning sulphur for preservation. During the XIX century, chemistry, biology and medicine advances, meant a great change for food preservation. In addition, the discovery of Louis Pasteur demonstrated that food spoilage was generated by microorganisms, which led to the development of inhibitors of their growth (Toussaint-Samat, 2009).

Nowadays, with the considerable increase of the world population, the changes of lifestyles and the consumer requirements, there was a need to promote the commercialization of food products. For this, it was necessary to increase the development of agricultural, industrial and technological techniques, which would allow a greater production and distribution of food in a safe, efficient and fast way (Winger and Wall, 2006; Carocho et al., 2014; Moragues-Faus et al., 2017; Sadowski and Baer-Nawrocka, 2018). This increase in production, combined with consumer's needs, required the development of foods with more favourable organoleptic characteristics and longer shelf life, giving rise to the discovery and application of food additives (Delgado-Vargas et al., 2000; Prado and Godoy, 2003; Carocho, et al., 2014; Asioli et al., 2017).

Thus, the application of additives aims to add dietary nutrition, increase the shelf life and/or improve the physical, chemical, sensory and microbiological properties of the industrialized food (Martins et al., 2017).

According to the Codex Alimentarius food additive is *"any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods"* (FAO/WHO, 2020).

Food additives are divided in different categories, such as colourants, preservatives, antioxidants, emulsifiers or stabilizers. They perform several functions depending of their purpose. For example, preservatives prevent food spoilage restricting the biological processes and antioxidants slow down or completely eliminate the oxidation reactions (Badora et al., 2019).

In the European Union, all these substances are classified according to the international numbering system (INS) for food additives, defined by the Codex Alimentarius Commission (Upadhyay and Pandey, 2012). The objective is to generalize the classification, becoming common in all the countries of the EU and, consequently, to facilitate its implementation, to monitor its regulation and also to guarantee more accessible information to consumers (Upadhyay and Pandey, 2012; Carocho et al., 2014).

The adopted nomenclature includes the letter "E" as a prefix, which represents Europe, and a 3-digit number that specifically identifies the food additive. The main classes of food additives are associated with a number, after the prefix E, so that: E1 represents the colourants, E2 the preservatives, E3 the antioxidants, E4 the texture agents, E6 the flavor intensifiers and E9 the sweeteners. The other existing classes are not included in any specific number (Carocho et al., 2014; Martins et al., 2016). For countries that do not belong to the EU, the labeling legislation is partially different. The classification used implies the same numbering, however, without the prefix “E” (Upadhyay and Pandey, 2012).

The monitoring of the use of these additives is carried out by the Joint Committee of Experts on Food Additives and Contaminants (JECFA) of the of the Food and Agriculture Organization (FAO) and World Health Organization (WHO), who evaluates the safety of the additives. It also recommends that all countries check the level of total consumption of each specific additive to ensure that its ingestion does not exceed the values determined in the Allowable Daily Dose (ADD) (Prado and Godoy, 2003; “Codex Alimentarius Commission-Procedural Manual,” n.d.; Upadhyay and Pandey, 2012).

In Europe, the permitted food additives are listed by their denomination and their respective ADD in the regulation 1129 of 2011 (European Parliament and Council, 2011). The values are defined and introduced by the JECFA (FAO and WHO, 2014). The ADD is an estimated value that refers to the amount of an additive that is present in a foodstuff, which

can be taken orally in a given daily dose without a considerable risk to health and is expressed in mg/kg (milligram of substance active per kilogram of body mass) (Branen et al., 2005).

In most countries outside the European Union (China, Egypt, Brazil), the control of food additives is governed by the Codex Alimentarius, which has the General Standard for Food Additives, CODEX STAN 192-1995.

In the United States, the Food and Drug Administration (FDA) is the organism that regulates food additives. In this sense, the Food Additives Status List of the FDA provides a brief knowledge on the use limitations for a food additive and the formally regulation could be found in the title "21 of the Code of Federal Regulations" (FDA, 2020).

For a substance to be approved for commercialization, it is essential that its consumption is monitored and reassessed in order to determine possible changes in its consumption margins (Branen et al., 2005; Martins and Ferreira, 2017).

Regarding the origin, the additives can be divided between natural and synthetic. Synthetic additives have the property of being more powerful and resistant to processing. These types of compounds are preferably used by the food industry, due to their lower cost. In addition, they provide products with specific taste, odour or colour and allow these characteristics to be stronger and last longer over time (Badora et al., 2019).

Otherwise, the natural additives are defined as substances present in food or extracted from a natural source. They are produced from plants or microorganisms extracts, after purification or enzymatic alterations. They are normally safer than those synthetically generated. The disadvantage of these additives is that they are usually more expensive and are needed in larger quantities than synthetics ones (Carocho et al., 2015). Moreover, they are unstable since their higher sensitivity to environmental factors, such as ambient temperature, oxygen content or sun exposure (Sharma, 2015).

In any case, toxicological, carcinogenic and safety tests must be carried out in order to guarantee their innocuousness (Carocho et al., 2015).

1.2.1 The particular case of colourants

Over the years, the visual appeal of food is as important as its taste or flavour. The visual appearance is the first sensation that generates expectations about the palatability of a food, a characteristic that is considered a crucial factor for the acceptance and consumption

of a product. Thus, the application of a colourant in a food product contributes, essentially, to guarantee a good appearance and to improve the organoleptic characteristics. Consequently, consumers interest and satisfaction would be ensured (Lin et al., 2018). These additives allow colour to be preserved over time (by inhibiting the discolouration as consequence of external factors: light, air, extreme temperatures and storage conditions); the correction of natural colour variations and the intensification of the natural colouring of a food product (Sharma, 2014; Amchova et al., 2015).

As previously mentoned, the entity responsible in the EU for the regulation of additives for food use is JECFA, while in the United States of America it is the FDA. These organizations define colourant as *"any dye, pigment or substance which when added or applied to a food, drug or cosmetic, or to the human body, is capable (alone or through reactions with other substances) of imparting colour*". The basic principles of regulation are to ensure that foods containing colouring substances are safe for consumption and to verify that only approved ingredients are listed as safe and used (Lewis, 1989; FDA, 2004; Sharma, 2014; Martins et al., 2016).

In order to add colour to a food product, manufacturers use inorganic, synthetic and natural colours. Inorganic colourants are mineral elements compounds. The most commonly used are titanium dioxide, aluminium and iron oxide. Titanium dioxide contributes as bright white colour and opacity, iron oxides are used to provide heat-stable red, yellow or black colours. Otherwise, synthetic colourants are preferably used in processed foods and beverages because they can be easily mixed to create a wide range of colours since they are stable under processing and storage conditions. Also, their production is less expensive than that of natural compounds. They have more shelf life, not or limited contribution to flavour and/or aroma in the finished product, high efficacy at low concentrations and superior chemical stability to processing and distribution (Corradini, 2018; Zeece, 2020). However, many studies have confirmed that excessive consumption of synthetic colourants has adverse effects on human health and are related to adverse reactions, such as gastrointestinal, respiratory, dermatological, neurological, increase the risk of getting cancer, allergies and may trigger hyperactivity in children (Carocho et al., 2014; Goren et al., 2015; Caleja et al., 2016). That is the reason why in recent years the food industry has been looking for safer and

more effective alternatives for replacing these synthetic additives with natural and healthier options (Corradini, 2018).

Natural colourants are organic substances derived from a plant, fruit or animal source (**Table 3**) and are an alternative more safety and healthy for the consumers. However, these compounds present some limitations that hinder their application at industrial level. Some of them are sensitive to environmental factors, such as pH, room temperature, oxygen content or sun exposure, making them not durable at the process or storage stages (Sharma, 2015). In addition, the applications of these natural pigments could be limited as consequence of their complex obtaining process, the lower stability at the application moment (changes in its colouring capacity may occur with the interaction with all food ingredients) and, finally, the availability of these natural resources is not the same in all countries (Patel, 2011).

Table 3. Natural food colourants.
(Source: Zeece, 2020)

COMPOUND	SOURCES	COLOUR
Anthocyanin	Grapes, berries, cabbage	Red, blue, purple
Betalain	Beetroot, amaranth	Red, purple
Caramel	Caramelized sugar (glucose, fructose, sucrose)	Amber, brown
Carminic acid	Cochineal insect	Carmine
Carotenoids	Annatto, beta carotene, lycopene, lutein	Yellow, orange, red
Chlorophy II	Spinach, broccoli	Green
Curcumin	Turmeric plant rhizome	Yellow, orange
Heme	Myoglobin, hemoglobin, leghemoglobin	Red, purple, brown
Minerals	Ilmenite (Titanium dioxide), coal tar, ethylene cracking tar (carbon black)	White, black, varied colours
Phycocyanin	Spirulina, cyanobacteria/algae	Green

Besides colourants classification according to their origin, natural ones can be categorized according to their colour (**Figure 1**): the green chlorophylls, the yellow-orange-red carotenoids and the red-blue-purple anthocyanins.

Chlorophyll is the component of plants, bacteria and algae responsible of their green colour. Even the prevalence of green tones in natural settings, this type of colour is one of the less investigated.

In the case of carotenoids, several forms have been identified. They are responsible of red, orange and yellow colours in many foods and flowers. Examples of foods with these compounds include carrot, pumpkin, tomato, paprika, saffron, marigold, among others. Some

animal foods, such as salmon and shellfish (lobster and shrimp), also contain carotenoids accumulated via the food chain from organisms that feed upon algae.

Anthocyanins belong to the flavonoid family of compounds that provides red, blue, purple and violet colours to a large number of plants, foods and flowers. They are the most extensively investigated members of the flavonoids group. Many berry-type fruits, red cabbage and purple sweet potato are recognized as good sources of anthocyanins (Rodriguez-Amaya, 2016; Sigurdson, 2017; Zeece, 2020).

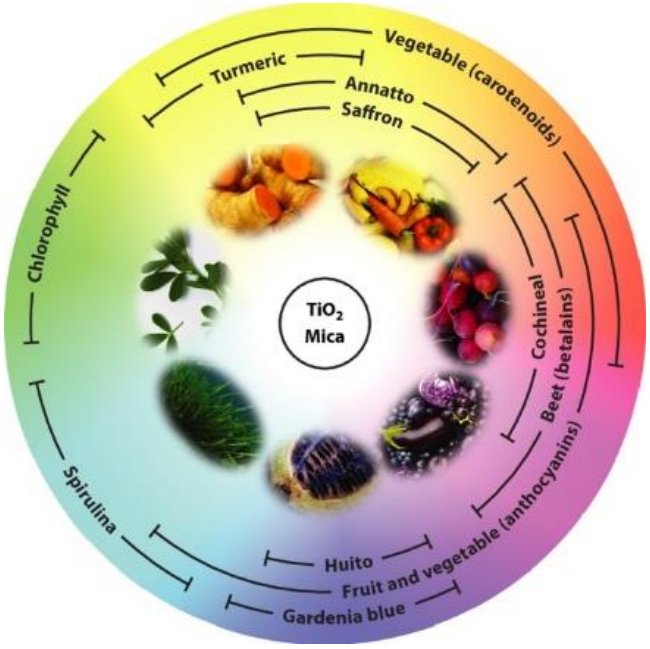


Figure 1. Representative food colourants from natural sources, organized according to their hue. (Source: Sigurdson et al., 2017).

1.2.2 Anthocyanins

In order to develop methodologies for isolate and stabilize colourants from natural matrices, several studies have been done. One of the most studied colourants classes is anthocyanins (Martins et al., 2016).

Anthocyanins belong to the flavonoid compounds family and provide red, blue, purple and violet colours to a large number of plants and flowers. Regarding the chemical characterization, they exist in the forms of aglycone (anthocyanidin) or glucoside (anthocyanin).

Anthocyanidins are grouped into 3-hydroxy anthocyanidins, 3-deoxy anthocyanidins, and *O*-methylated anthocyanidins. While anthocyanins are in the forms of anthocyanidin glycosides and acylated anthocyanins. The most commonly found compounds are in the forms of aglycone, including pelargonidine, cyanidin, delphinidin, peonidine, malvidin and petunidine.

The differences between anthocyanins consist in the number of hydroxyl groups, their nature, the number of sugar compounds and the position of these attachments, as shown in **Figure 2** and **Table 4**. Anthocyanin colours are derived from the excitation by visible light of double bond electrons in their structure (He and Giusti, 2010; Khoo, 2017).

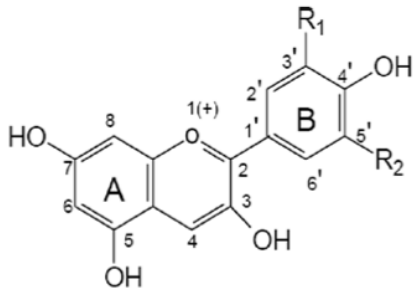


Figure 2. Basic chemical structure of anthocyanidins.
(Source: He and Giusti, 2010).

Table 4. Substituents of anthocyanidins in **Figure 2**.
(Source: He and Giusti, 2010).

AGLYCONE	SUBSTITUENTS	
	R1	**R2**
Pelargonidin (Pg)	H	H
Cyanidin (Cy)	OH	H
Delphinidin (Dp)	OH	OH
Peonidine (Pn)	OCH_3	H
Malvidin (Mv)	OCH_3	OCH_3
Petunidine (Pt)	OCH_3	OH

Some sources of these compounds available in the nature are berries, cherries, peaches, grapes, pomegranates and plums, as well as many dark-coloured vegetables such as black currant, red onion, red radish, black bean, eggplant, purple corn, red cabbage and sweet purple potato (Wu et al., 2006). Although, they are most commonly accumulated in flowers and fruits, present in leaves, stems and storage organs (Delgado-Vargas and Paredes-Lopez, 2003).

Another important characteristic of anthocyanins, is their beneficial role for health. These molecules are described as therapeutic compounds, namely in the reduction of coronary heart disease, cancer, diabetes. They also exhibit anti-inflammatory effects and improvements of visual acuity and cognitive behaviour (Li et al., 2017; Leong et al., 2018; You et al., 2018).

However, despite the excellent colouration of anthocyanins, they are sensitive to numerous variables, such as light, pH, temperature, humidity, salinity and stress conditions. The types of anthocyanins depend on the pH of the solution. Some research has determined that at low values they are more stable than in alkaline solutions (Rein, 2005). Light accelerates their degradation and at higher sugar concentrations, the negative effect is greater. At high temperatures, darker products are obtained, so it is recommended to keep these compounds at low temperatures. Also, the combination of high temperatures and oxygen presence is a factor of great impact in their degradation. Another factor that causes discolouration are the sulfates and sulfites used for better storage (Castaneda-Ovando, 2009; Turturică et al., 2015; Pervaiz et al., 2017).

1.2.2.1. Anthocyanin extraction

Extraction is the most critical step to obtain a natural colourant. It depends on the characteristics of the natural matrix and the factors that impact the process, such as temperature, parts of the plant, pressure and solvent type (Hernández et. al, 2009). In this way, with regard to extraction methods, these can be varied depending on the natural matrix and the final objective of the work. There are several types of extraction methods, some of them are supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave assisted extraction (MAE), ultrasound assisted extraction (UAE), pulsed electric field extraction (PEF) and enzyme assisted extraction (EAE) (Azmir et al., 2013).

In the microwave-assisted extraction (MAE), the electromagnetic field of microwaves ranges from 300 MHz to 300 GHz, which are composed of two perpendicular fields: magnetic and electric. The heating principle of microwaves is based on its immediate effects on polar materials (Letellier and Budzinski, 1999). Some of the benefits of this technique are: lower equipment sizes, higher extract amounts, less extraction time and higher quality of the extract, since thermolabile compounds are not considerable affected during the process

(Cravotto et al., 2008). It is considered as a green method, since it reduces the use of organic solvents. Besides, the bioactive compounds could be extract faster and more efficiently than conventional technologies (Alupului et al., 2012).

Meanwhile, the principle of pulsed electric field (PEF) is to increase the extraction by breaking the structure of cell membranes. Electric charge splits the molecules of the cell membranes due to their dipole nature (Ade-Omowaye et al., 2001). This technique minimizes the degradation of heat-labile compounds, because an optimum electric field prevents temperature increase, which has a little impact on plant cell membranes (Fincan and Dejmek, 2002).

The Enzyme-assisted extraction (EAE) can be performed in two ways, one is enzyme-assisted cold pressing (EACP) and the second is enzyme-assisted aqueous extraction (EAAE) (Latif and Anwar, 2009). The last one has been developed for the extraction of oils from different seeds (Hanmoungjai et al., 2001). Some benefits of these methods are that the operation time is shorter, more quantity and quality of bioactive compounds could be obtained and water is used as solvent instead of organic ones. So, it is considered ecofriendly (Puri et al., 2012).

Liquid-liquid extraction is a method in which one or more solutions are added to a liquid feeding solution that is completely mixed with the solvent, whereby a solute is transferred from one to the other. This technique is useful to extract phenolic compounds. A benefit of liquid-liquid extraction is that industrial by-products can be reused, like liquids from the beverage industry, since they are rich in bioactive compounds (Müller et al., 2008).

On the other hand, heat-assisted extraction (HAE), known as maceration, is easy to apply and relatively inexpensive (Azmir et al., 2013). This method provides a solution to small molecules that could be quickly isolate in a compatible solvent, without the need of cleaning the sample, thereby avoiding pre-concentration of the sample. Also, it allows samples to be analysed immediately (Linz, 2013).

Finally, the super critical fluid extraction is considered a green and clean technique for the extraction of bioactive components from waste matrices. One of the most used solvents is CO_2 because it is cheap, safe and easy to recycle and gives cleaner extracts than the conventional extraction. The benefits of using this type of solvent are: moderate critical conditions of temperature (31°C) and pressure (73.8 MPa), non-toxic nature and high

chemical stability. Furthermore, CO_2 penetrates quickly into the food matrix, since it has a higher diffusion coefficient and a lower viscosity and surface tension. Therefore, extraction time is reduced (Lang and Wai, 2001; Azmir et al., 2013).

The anthocyanins chemical stabilization is the main focus of recent studies due to their abundant and potential applications, their beneficial effects and their use as alternative to artificial colourants. For anthocyanins recovery, several factors that affect their stability must be considered. The most important are pH variations, high temperatures and the presence of light (Ngamwonglumlert et al., 2015).

The colour of anthocyanins depends on the pH of the solution since their molecular structure has an ionic nature (Turturică, 2015). It is suggested to use them at low pH (<4.0), since they are in the form of flavylium cations that are more stable than other structures (Tan et al., 2014).

Regardless of the chosen extraction procedure, its optimization is essential, since normally, the optimization parameters focus on the solvent and the extraction time/temperature ratio (Martín et al., 2017). Thus, the choice of the solvent must be based on the chemical structure of the anthocyanins, which are made up of non-polar aglycones, where the substitutions in the aromatic rings are mostly polar. In this sense, the solvent should preferably be an alcohol/water mixture (Martín et al., 2017). The most commonly used alcohol is ethanol, because it is considered not toxic in the food industry or in clinical practices. Methanol, acetone and acetonitrile can also be employed. In addition, the extraction solvent must be sufficiently acidified, so that partial hydrolysis of the anthocyanin acyl fractions does not occur and to maintain their flavyl cation form (red colour), which is the most stable form of anthocyanins (Flores et al., 2016; Teng et al., 2017). For this, in order to adjust the pH of anthocyanins, hydrochloric, citric, malic, ascorbic, tartaric and acetic acids are used (Ongkowijoyo et al., 2018).

Considering the extraction time/temperature ratio, a higher quantity of anthocyanins is obtained when extractions are performed in a short time, depending on the type of methodology applied. In order to avoid anthocyanins degradation in long time extractions (for higher extraction yield), room temperature should be employed (Flores et al., 2016). However, this principle is not linear, since the application of moderate temperatures at short times can increase the extraction yield of these compounds.

HAE was selected as the best extraction method for this study, since it is one of the simplest, easier to apply, relatively inexpensive and promotes the extraction of bioactive compounds using organic solvents or water/alcohol mixtures.

In order to assess the influence of these three variables (time, temperature and solvent) on the extraction yield of anthocyanins from natural matrices, the response surface methodology has been used (RSM). This is a statistical tool used in the optimization of extraction processes, essential at the industrial level as it guarantees the conditions in order to maximize the extraction yield with the least expenditure of time and reagents, predicting the optimal conditions and interactions between the different variables (Alexandre et al., 2017b, 2017a).

1.3 *Malpighia emarginata* DC.

1.3.1 Etymology and applications

The plant *M. emarginata* (**Figure 3**) belong to the *Malpighiaceae* family and is a tropical specie from the Caribbean region, being native from the West Indies and tropical America. Due to the morphological similarity with the European cherry, this specie is called "West Indian Cherry". However, this fruit is popularly known as "acerola", but was given different names depending on the country where it is grown, being known as "Barbados cherry", "cerise des Antilles" and "sweet kersie" (Oliveira et al., 2012; Moreira et al., 2016). Nowadays, this fruit is cultivated in several places, namely in tropics and in subtropical areas, such as Florida, Australia and Israel, but also can be found from South Texas, through Mexico and Central America (**Figure 4**). More recently, it has been introduced in subtropical areas throughout the world, such as parts of Asia, India and South America (de Assis et al., 2008).

Figure 3. *Malpighia emarginata* DC.
(Source: http://www.unirio.br/ccbs/ibio/herbariohuni/malpighia-emarginata-dc)

Figure 4. Geographic representation of *M. emarginata* producing countries (Source: de Assis et al., 2008).

Nowadays Brazil is the largest producer of this fruit, specifically the Northern region of the country, with 11000 hectares of plantation, producing 3000 kg/ha and a total of 32990 tons/year (Pommer and Barbosa, 2009). This country has also dominated the marketing and exporting of processed products from this fruit, like frozen fruit, juice, marmalade, frozen concentrate, jam and liquor (Delva and Schneider, 2013a). However, this fruit is also cultivated at small scale in others countries. In France, Germany and Hungary, *M. emarginata* is used largely by food industry in juice form (Delva and Schneider, 2013b).

This matrix, in addition to its application in the food sector, also exhibits therapeutic characteristics of high interest. In folk medicine, acerola has a great attractive due to its high vitamin C content. In addition, its ingestion is recommended for the management of several diseases caused by oxidative stress, considering its antioxidant activity. Pharmacological activities have been reported for *M. emarginata* fruits, natural juices and industrial pulps and their beneficial effects are attributed to low molecular weight compounds such as ascorbic acid, malic acid, tartaric acid (Klosterhoff et al., 2018).

Additionally, this specie is widely used in popular culture as a medicinal plant for the treatment of symptoms related to respiratory, cardiovascular and cholesterol diseases. Although several communities and populations use acerola juice mainly for the treatment of respiratory diseases, some reports have shown that *M. emarginata* leaves are also used to make tea and syrup in the treatment of influenza symptoms and to treat cholesterol and hypertension (Da Silva Barros et al., 2020). In the United States it is utilized by the

supplement and pharmaceutical industries as a rich source of ascorbic acid (Delva and Schneider, 2013b).

This fruit has other phytochemical constituents such as flavonoids, phenolic acids and polyphenols (Bataglion et al., 2015; Malegori et al., 2017), that allows the beneficial incorporation of its extracts in several industrial sectors, specifically, food, pharmaceutical and cosmetic. This is possible, because these compounds interfere beneficially in human health, minimizing the oxidative damage caused by the reactive oxygen and nitrogen species in organism (Oliveira et al. 2012) and prevent some chronic diseases such as cancer (Leffa et al., 2014). These compounds also exhibit potential therapeutic effects as anti-inflammatory, radiation-protective, chemoprotective, vasoprotective, LDL oxidation inhibitor and decreasing the risks of cardiovascular diseases (Bolson et al., 2015; Beltreschi et al., 2018).

In this sense, considering the increase in the production of functional food based in new technologies, *M. emarginata* seems to be a good candidate for the production of both foods and medicinal products. Acerola skin is a by-product of its processing, usually discarded in juice and pulp industries. The experimental data compiled have shown that ascorbic acid, phenolic compounds and carotenoids are key compounds in the chemical composition and the biological activity of *M. emarginata* fruit. One of the most important are the anthocyanins, that provide most of the red, blue and purple colours. Their visual impact allied to their health benefits make them potentially attractive as natural food colourants. Thus, the objective of this work is to isolate and characterize the phenolic compounds and estimate the amount of anthocyanin pigments present in *M. emarginata* fruits.

1.3.2 Chemical composition and bioactive properties

M. emarginata is a specie with a great economic importance since it is largely appreciated and consumed, not only as natural, but also as industrialized forms (juices, jellies, concentrates, ice creams, syrups, liqueurs, fruit syrups, among others). Its demand has grown, principally due to its high concentration of bioactive and antioxidant compounds, such as: ascorbic acid, bioflavonoids and phenolic compounds. It should be mentioned that the most common form of acerola consumption is as industrialized products, generating large volumes of organic waste (approximately 40% vol) (Marques et al., 2009).

However, the extraction and processing of the promising compounds present in the acerola residue could increase the commercial value of this raw material and its profitability. Several studies have investigated how to characterize the *M. emarginata* (leaves, fruits and stem), detailing its taxonomic, vegetative, phytochemical, nutritional and biological composition (Delva and Schneider, 2013b; Jaeschke et al., 2016; Yara et al.,2017; da Silva Barros et al., 2020).

Chemical and nutritional characteristics of the *M. emarginata* fruit are affected by several factors: climatic conditions (rainfall, availability of soil nutrients, temperature variation), cultivation methods, geographic location, pesticides application, maturation stage, processing and storage.

Bearing in mind the high consumer interest in the nutritional and bioactive properties of *M. emarginata* and its potential for therapeutic applications, several studies have been carried out by the scientific community in order to determine the nutritional, chemical and bioactive characteristics of the plant and fruit.

The fruit of *M. emarginata* is composed by a greater proportion of water (90.6-92 g/100g) and several macro and micronutrients, which are summarized in **Table 5**. Glucose (2.14-3.33 g/100g), fructose (0.25-0.38 g) and small amount of sucrose (0.02 g/100g), as well as, low fat (0.23-0.80 g/100g) and carbohydrates concentrations (3.57-7.8 g/100g) could be detected. Other found compounds are malic and tartaric acids, which are present in small amounts. In the micronutrients group, vitamin C, as the main vitamin (695-4827 mg/100g), and minerals at lower concentrations, could be mentioned. Vitamin C plays a significant role in the human nutrition because it is well absorbed by the body. Also in the industry, since it could be use as concentrate for pharmaceutical purposes or for industrialized foods enrichment.

Table 5. Chemical and nutritional composition of *M. emarginata*
(Source: Delva and Schneider, 2013b)

CHEMICAL AND NUTRITIONAL COMPOUNDS		CONTENT (in 100 g *M. emarginata* fruit)
Water		90.6-92 g
Protein		0.21-0.80 g
Fat		0.23-0.80 g
Carbohydrate		3.57-7.8 g
Sugars	Fructose	0.25-0.38 g
	Glucose	2.14-3.33 g
	Sucrose	0.02 g
Vitamins	Vitamin C	695-4827 mg
	Vitamin B6	8.70 mg
	Vitamin B2	0.07 mg
	Vitamin B1	0.02 mg
Minerals	Phosphorus	17.1 mg
	Calcium	11.7 mg
	Iron	0.22 mg
	Ash	0.40 g
Dietary fiber		3 g
Soluble solid		7.70-9.20 g
Organic acids	Malic acid	0.25-0.38 g
	Citric acid	0.01-0.03 g
	Tartaric acid	0.002-0.01 g

In addition to their composition in nutrients, these fruits are composed by non-nutrients (**Table 6**), being the phenolic compounds the most abundant. These types of compounds are considered as bioactive components that are associated with the prevention and co-adjuvant of many diseases. Delva and Schneider (2013b) analysed the available information regarding the production, postharvest handling, chemistry, nutrition and potential biological activity of *M. emarginata* fruit. Their results evidenced the presence of several compounds with functional action. Two bioactive groups are found in greater proportion: phenolics (536-4524 mg), being anthocyanins (3.79-59.74 mg) and flavonoids (7-18.5 mg) the most important, and in small proportion, carotenoids with a total of 0.32-40.6 mg. From the last group, α-carotene (1.6-3.6 mg) has the highest percentage and β-carotene and lutein, with 0.5-1.2 mg and 0.1-3.6 mg, respectively.

Table 6. Bioactive composition of *M. emarginata* (Source: Delva and Goodrich Schneider, 2013b.)

BIOACTIVE COMPOUND	**CONTENT (in 100 g *M. emarginata* fruit)**
Total phenolics	536-4524 mg
Anthocyanins	3.79–59.74 mg
Flavonoids	7-18.5 mg
Total carotenoids	0.32-40.6 mg
β-Carotene	0.5-1.2 mg
α-Carotene	1.6-3.6 mg
Lutein	0.1-3.6 mg

According to the information available in the literature, several studies have proven that these bioactive compounds are connected to both nutritional (reduction of oxidative stress, prevention of cancer, arteriosclerosis and aging processes) and functional properties (preservative of vegetable oils and emulsions) (Galanakis, 2017). They exhibit beneficial effects such as antioxidant activity, inhibition or induction of enzymes, inhibition of receptor activities and induction or inhibition of gene expression (Correia et al., 2012).

Objectives

2. Objectives

Considering the importance attributed to bioresidues from the food industry, due to its high content of bioactive compounds and the demand for the implementation of new natural ingredients in food products, this work aims to characterize the bioresidues of *M. emarginata* fruits, regarding their nutritional and chemical composition, as well as, the bioactive action of the hydroethanolic extract.

Therefore, the specific objectives are:

- Determination of nutritional composition through official AOAC analysis methodologies, analyzing the protein, ash, fat, carbohydrate and energy content;
- Determination of chemical composition using chromatographic techniques coupled to different detectors, analyzing free sugars, fatty acids, organic acids and phenolic compounds (anthocyanin and non-anthocyanin compounds);
- Determination of the bioactive potential of the fruit of *M. emarginata* by the evaluation of antioxidant, cytotoxic, anti-inflammatory and antimicrobial activities;
- Optimization of anthocyanins extraction from *M. emarginata* fruits, carried out through the application of heat assisted maceration, applying the response surface analysis (RSM) method.

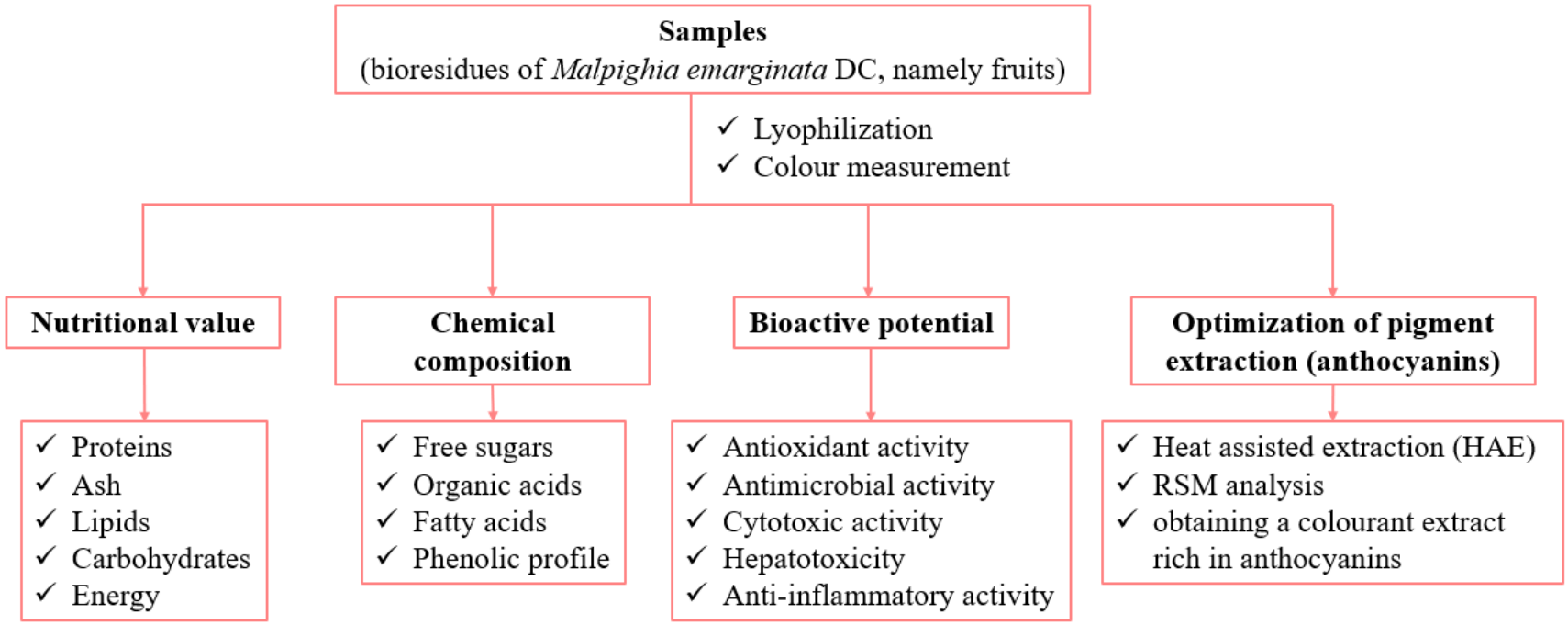


Figure 5. Representative scheme of the mainely objectives and procedures developed in this dissertation.

Material and Methods

3. Methodology

3.1. Samples acquisition and preparation

The bioresidues of *M. emarginata* used for this study, were obtained fresh from a Brazilian company, in September of 2020. In this study, all fruits that had no commercial value, for example, fruits that did not fulfill the necessary physical requirements for their commercialization, were considered bioresidues (**Figure 6**).

After acquiring the samples (1 kg), the stems were removed by a mechanical process. Subsequently, the entire prepared samples were frozen and dehydrated through a freeze-drying process (FreeZone 4.5, Labconco, Kansas City, MO, EUA). After this treatment, the dried samples were shipped to Portugal from Brazil. In CIMO (Portugal), the acerola samples were reduced to a fine dried powder and then stored in a cool, dry place away from light, to subsequently carry out the nutritional, chemical and bioactive analyses and the optimization processes.

Figure 6. *M. emarginata* fruits in fresh form (A) in lyophilization process (B) and lyophilized (C). (Source: Fernanda Leimann and from the author).

3.2. Standards and reagents

3.2.1. Nutritional value, chemical analysis and optimization studies

The solvents n-hexan 95%, acetonitril 99%, etanol absolut (99,9%) and ethyl acetate 99,98%, all of HPLC grade, were acquired in the company Fisher Scientific (Lisbon, Portugal). The analytical grade methanol solvent was purchased to Paralab company (Lisbon, Portugal). The remaining solvents used, also of analytical grade, were the following: ethyl ether (Lab-Scan, Lisbon, Portugal); toluene and sulfuric acid (Sigma-Aldrich; St. Louis, MO,

EUA). Water was treated prior to its use by the Milli-Q-Water purification system (TGI Pure Water Systems, Greenville, SC, EUA).

The standard blend of 37 fatty acid methyl esters (FAME) (C4-C24; standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, MO, EUA), as well as the sugar standards (D (-)-fructose, D(+)-sucrose, D(+)-glucose, D(+)-trehalose, D(+)-melezitosis and D(+)-raffinose pentahydrate), and organic acid patterns (L(+)-ascorbic acid; citric acid; malic acid; oxalic acid; succinic acid; fumaric acid and quinic acid). Regarding the standards of the non-anthocyanin phenolic compounds used (apigenin-6-*C*-glucoside, caffeic acid, ellagic acid, chlorogenic acid, hesperetin, luteolin-7-*O*-glucoside, naringenin, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, taxifoline, rosmarinic acid), they were purchased from Extrasyntheses (Genay Cedex, France). Besides, the anthocyanin compounds (cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside) were acquired at Polyphenols (Sandines, Norway).

3.2.2. Study of bioactivities

i) **Analysis of cytotoxic and hepatotoxic activity:** Fetal bovine serum (SFB), L-glutamine, Hank's saline solution (HBSS), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) and DMEM medium (animal cell culture medium (Dulbecco Modified Eagle)) were purchased from Hyclone (Logan, Utah, EUA). Acetic acid, ellipticin, sulforodamine B (SRB), trichloroacetic acid (TCA) and Tris were supplied by Sigma-Aldrich (St. Louis, MO, USA). Water was treated as previously mentioned in ***section 3.2.1.***

ii) **Anti-inflammatory activity:** RAW 264.7 cells were purchased from ECACC ("European Collection of Animal Cell Culture") (Salisbury, UK) and DMEM from Hyclone (Logan, Utah, EUA). The Griess Reagent System Kit was purchased from Promega (Madison, WI, EUA).

iii) **Analysis of antimicrobial activity:** Mueller-Hinton agar (MHB) was obtained from Biolab® (Hungary). The compound p-iodonitrotetrazolium chloride (INT) was purchased from Panreac Applichem (Barcelona, Spain). The antibiotics imipenem and vancomycin were obtained from Hikma pharmaceutical (Portugal SA) and Ampicillin from pharmaceutical Janssen (Belgium).

iv) **Analysis of antioxidant activity:** The trolox standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and AAPH were provided by Sigma-Aldrich (St. Louis, MO, EUA).

3.3. Evaluation of the colour in the epicarp of *Malpighia emarginata* DC. fruits

The colour parameters in the epicarp of *M. emarginata* fruits were evaluated as previously described by Roriz et al. (2017). The colour was measured in whole and crushed lyophilized samples by a colorimeter (model CR-400, Konica Minolta Sensing, Inc., Osaka, Japan) with an adapter for granular materials (model CR-A50). The measurements were made in the CIE *L*a*b** colour space using the illuminate C and a diaphragm aperture of 8 mm (**Figure 7**). *L** values represent brightness, where *L** = 0 produces black and *L** = 100 indicates diffuse white, negative values of *a** represent green while positive indicate magenta; and negative values of *b** represent blue and positive values indicate yellow. Data were processed with the "Spectra Magic Nx" (version CM-S100W 2.03.0006) software, from Konica Minolta.

Before starting measurements, the instrument was calibrated employing a standard white tile. For this purpose, three samples of epicarps fruits were selected and three measurements were performed on each sample (**Figure 8**).

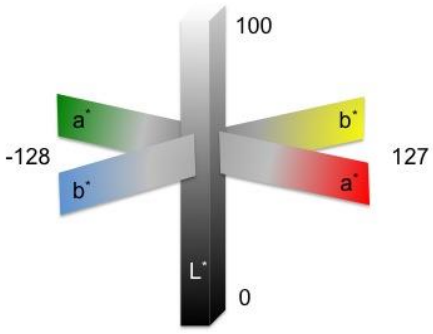


Figure 7. CIE L* a* b* model
(Source: Bora et al., 2015).

Figure 8. Measurement of the colour parameters in whole and crushed lyophilized fruits of *M. emarginata*. (Source: from the author).

3.4. Determination of nutritional value of the *Malpighia emarginata* DC. fruits

For the nutritional evaluation, the macronutrient composition of the *M. emarginata* fruits was determined using official food analysis methodologies (AOAC, 2016). Protein content, fats, carbohydrates, moisture and energetic value were analysed (**Figure 9**).

The protein content (N×6.25) was obtained by the macro-Kjeldahl method (AOAC 991.02) (**Figure 9A**), which is based on the amount of nitrogen present in the sample. To this end, concentrated sulfuric acid (H_2SO_4) was added to the sample (0.5 g). So, organic matter was digested and ammonium sulfate $(NH_4)_2SO_4$ was formed. Nitrogen was thus retained as an inorganic salt. Sodium hydroxide (NaOH) was then added in order to alkalize the solution and turn nitrogen in ammonia, NH_3. The ammonia was then distilled and collected in a sulfuric acid solution (H_2SO_4, 0.1 M). Finally, a titration with NaOH (0.1 M) was done, using a methyl red indicator, which allows quantifying the nitrogen present in the sample.

The lipids (**Figure 9B**) were determined by extracting a known sample mass (3 g) in a Soxhlet apparatus. Petroleum ether was used as the extraction solvent at a temperature of approximately 80ºC for 7 hours (AOAC 989.05).

To obtain the ash content (**Figure 9C**), incineration was carried out at 550 ± 15°C, for 12 hours, and the remanent solid was weight (AOAC 935.42).

To determine the moisture of the sample, a known quantity of sample (1 g) was placed in an oven (105ºC) until a constant weight was obtained.

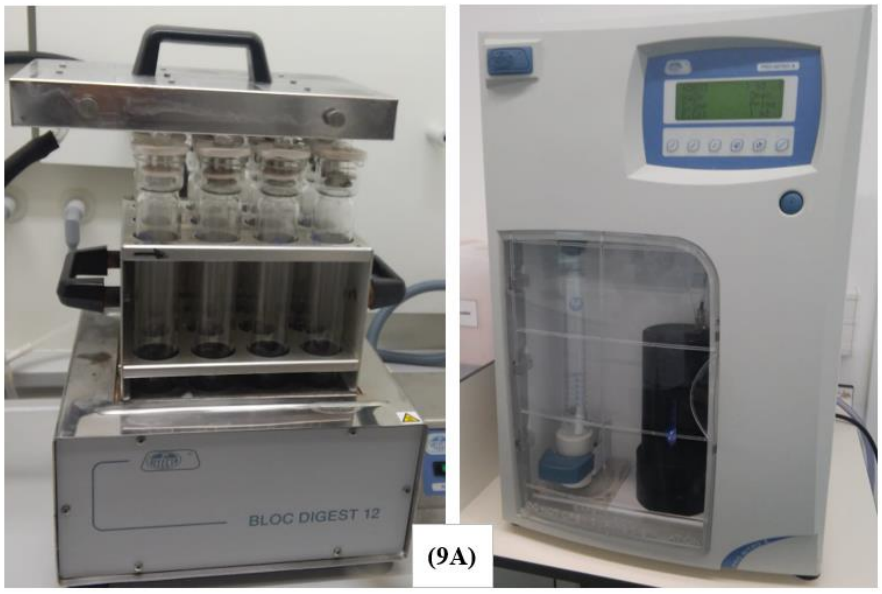
Finaly, carbohydrates were calculated by difference (**Equation 1**), while total energy was determined according to **Equation 2**.

$$Carbohydrates = 100 - (g\ proteins + g\ lipids + g\ ash)$$

Equation 1. Equation for determining carbohydrates.

$$Energy\ (Kcal) = 4 \times (g\ protein + g\ carbohydrates) + (9 \times g\ fats)$$

Equation 2. Equation for determining total energy.



(9A)

(9B)

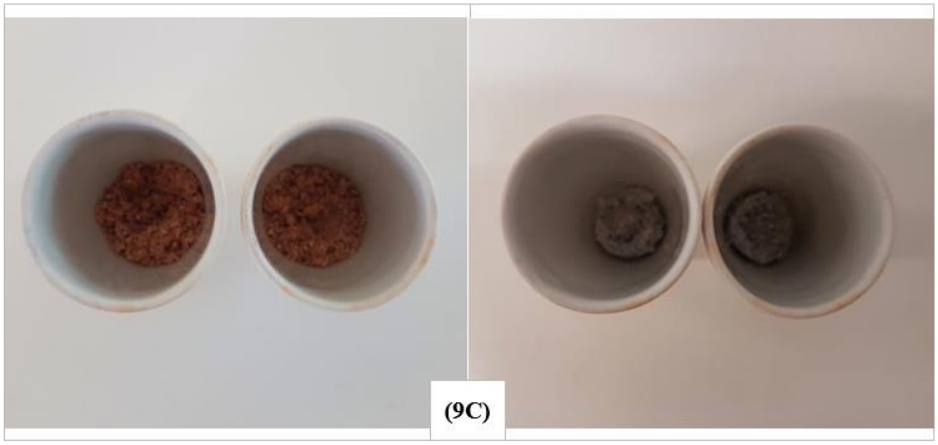


Figure 9. Tests performed for the evaluation of different parameters of nutritional value: proteins (A), lipids (B) and ash (C).
(Source: from the author).

3.5. Determination of chemical composition of the *Malpighia emarginata* DC. fruits

3.5.1. Free sugars

The free sugars were determined by high performance liquid chromatography coupled to a refractive index detector (HPLC-RI), as previously described by Barros et al. (2013).

In this assay, 40 mL of ethanol (80:20, *v/v*) were added to the sample (1 g) and the mixture was maintained at 80°C in a bath (Julabo, SW22; Seelbach, Germany) for 1 hour and 30 min. Stirring was done every 15 minutes.

Subsequently, the supernatant obtained was centrifuged (K24OR refrigerated centrifuge, Centurion, West Sussex, United Kingdom) at 5000 *rpm* for 10 minutes and then evaporated the ethanolic fraction, using a rotary evaporator (Büchi R-210, Flawil, Switzerland) at 40ºC under vacuum. The aqueous phase was washed 3 times with diethyl ether (10 ml) (**Figure 10A**), after which the remains were evaporated. To the dry residue obtained (**Figure 10B**), water was added to a final volume of 5 mL. Finaly, 1.5 mL were filtered (nylon filters 0.2 μm, Whatman) into a vial, in order to proceed with the sugars profile analysis in the HPLC system.

The HPLC system was equipped with a pump (Knauer, Smartline 1000 System, Berlin, Germany), a degassing system (Smartline manager 5000), an automatic sampler (AS-2057 Jasco, Easton, Maryland, EUA) and a detector of refractive index (Knauer Smartline 2300). The chromatographic separation was obtained through a Eurospher 100-5 NH2 column (4.6 x 250 mm, 5 μm, Knauer), which operated at a temperature of 35ºC (7971 R Grace).

The mobile phase used was acetonitrile/deionized water (70:30; *v/v*) with a flow rate of 1 mL/min. The injection volume was 10 μL. To identify the compounds, Clarity 2.4 Software (DataApex) was employed. Each relative retention time of the peaks in the chromatogram was compared with known standards. Melezitose was used as an internal standard (25 mg/mL). The quantification was made by the internal standard method and expressed in grams of compound per 100 g of fresh weight.

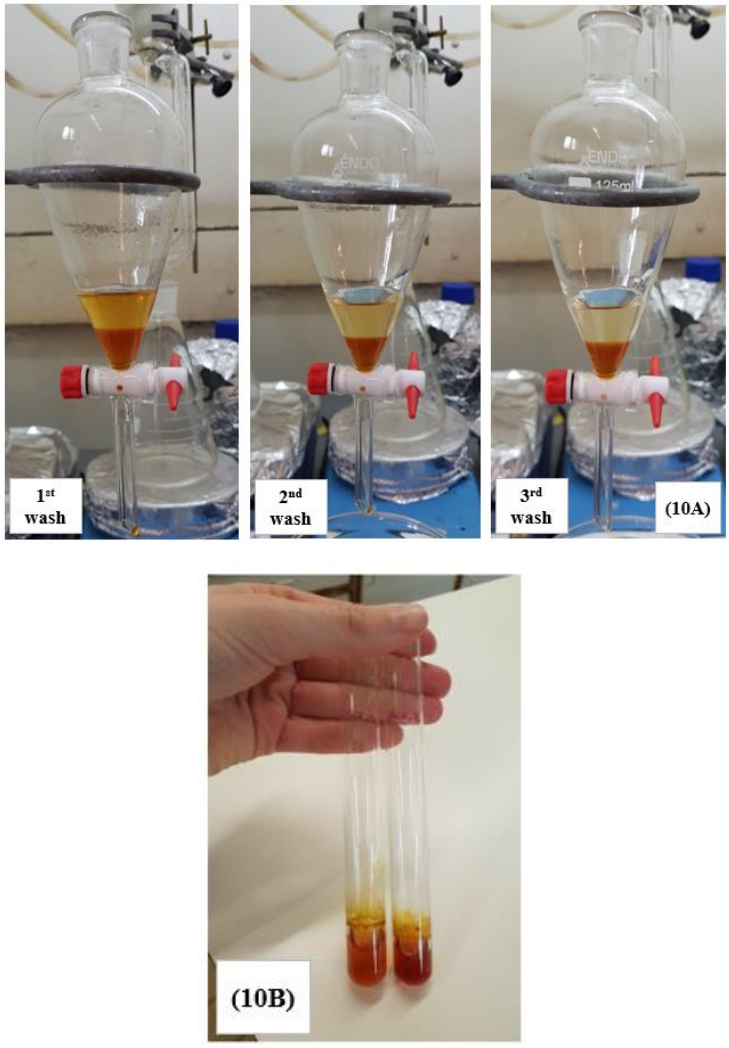


Figure 10. Procedure used to the evaluation of free sugar profile: aqueous phase washing process (A) and dry residue obtained after evaporation, with water to a final volume of 5 mL (B).
(Source: from the author).

3.5.2. Organic acids

Organic acids were determined using high performance liquid chromatography coupled to a diode detector (UFLC-DAD), according to a procedure previously described by Barros et al. (2013).

The extraction was carried out in the dark by adding 25 mL of metaphosphoric acid (4.5%) to the sample (1.5 g), maintaining the temperature conditions of 25ºC and constant agitation (150 *rpm*) for 45 minutes. After extraction, the mixture was filtered through filter paper (Whatman N°4) and then through nylon filters (0.2 μm; Whatman), namely 1.5 mL were filtered into a vial for analysis at UFLC (Shimadzu Corporation, Kyoto, Japan). The compounds separation was obtained through a SphereClone column (Phenomenex, Torrance, California, EUA) of C18 reverse phase (5 μm, 250 mm x 4.6 mm id) thermostated at 35°C. The elution was carried out with sulfuric acid (3.6 mM) with a flow rate of 0.8 mL/min and with an injection volume of 20 μL (**Figure 11**).

The detection of organic acids was achieved using a DAD system, applying the wavelengths of 215 nm and 245 nm (for ascorbic acid). The quantification of the compounds was performed by comparing the area of their recorded peaks at those wavelengths, with the calibration curves obtained from the standards of the respective compound. The results were expressed in mg of compound per 100 g of fresh weight.

Figure 11. Procedure used for the evaluation of the organic acids profile.
(Source: from the author).

3.5.3. Fatty acids

Fatty acids were determined by gas chromatography with flame ionization detection (GC-FID), as previously described by Pereira et al. (2012).

A solution of methanol/sulfuric acid/toluene (5 mL) was added in the lipid extract previously obtained by extraction in a Soxhlet (***Section 3.4***). The mixture proportions were 2:1:1 (*v/v/v*). The obtained solution remained in a bath (Julabo, SW22; Seelbach, Germany) at 50°C (with 160 *rpm* agitation) for, approximately, 12 hours. In order to enhance the phase separation, after removing the tubes from the bath, deionized water (3 mL) was added to the mixture. Subsequently, diethyl ether (3 mL) was incorporated to recover the fatty acid methyl esters (FAME). After phase separation, the supernatant was transferred to a vial, in which anhydrous sodium sulfate was previously added in order to dehydrate the supernatant. Finally, it was filtered through nylon filters (0.2 μm; Whatman; Sigma-Aldrich, St. Louis, MO, EUA) to a vial, for later analysis in GC (**Figure 12**).

The fatty acid profile was obtained through a GC system (Model DANI GC 1000) equipped with a split/splitless injector, a flame ionization detector (FID, 260ºC) and a Zebron-Kame column (30 mx 0.25 mm ID × 0.20 μm df; Phenomenex, Lisbon, Portugal). The temperature program applied was as follows: 100°C as initial temperature, for 2 min; progressive temperature increase: 10°C/min up to 140°C; 3°C/min to 190°C; 30°C/min to 260°C which remained for 2 min. The carrier gas used was hydrogen with a flow rate of 1.1 ml/min, measured at 100°C. The split injection (1:50) was performed at 250°C, where 1 μL of the sample was injected.

The identification of fatty acids was made based on the relative retention times of the FAME peaks in the samples in comparison with known standards. For processing the results, the CSW 1.7 software (DataApex 1.7, Prague, Czech Republic) was used and these were expressed as a relative percentage (%) for each fatty acid detected.

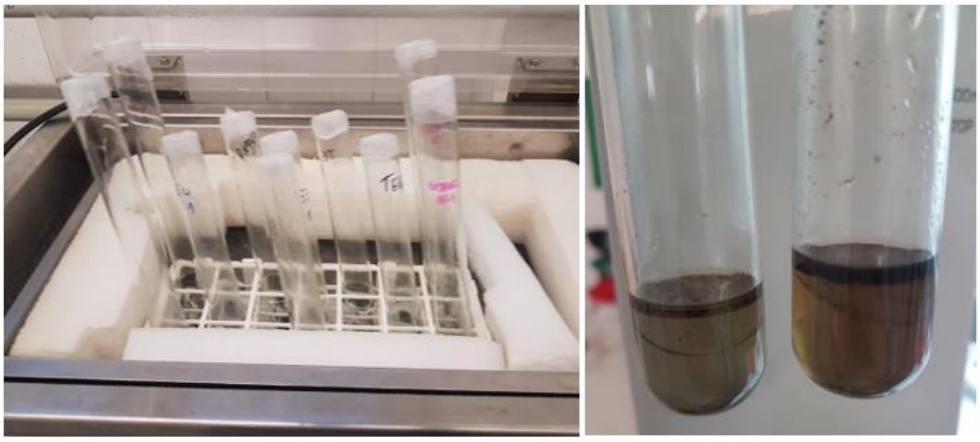


Figure 12. Procedure used for the evaluation of fatty acids profile.
(Source: from the author).

3.5.4. Phenolic compounds

The analysis of anthocyanin and non-anthocyanin phenolic compounds was performed on the entire fruit of *M. emarginata*, due to its red coloration. To this end, the methodology previously described by Bessada et al. (2016) and Gonçalves et al. (2017) was followed.

3.5.4.1. Non-anthocyanin phenolic compounds

Extraction procedure. An extract was prepared from the dry fruit material, previously dehydrated by lyophilization. The lyophilized sample (1 g of *M. emarginata* fruits) was subjected to maceration with an ethanol/water solution (80:20, *v/v*; 30 mL) at room temperature under constant agitation (150 *rpm*) during 1 hour. Subsequently, it was filtered through filter paper (Whatman N°4; Sigma-Aldrich, St. Louis, MO, EUA) and the process was repeated in the re-extracted with 30 mL of the same hydroethanolic solution. Finally, the alcoholic fraction of the obtained extracts was evaporated under vacuum (Büchi R-210, Flawil, Switzerland) and the aqueous fraction was lyophilized (47°C, 0.045 bar; FreeZone 4.5, Labconco, Kansas City, MO, EUA) to further analysis. A quantity of the obtained dry extract (10 mg) was subsequently re-dissolved in an EtOH/H_2O solution (20:80, *v/v*; 2 mL) and then filtered through 0.22 μm LC disposable discs for further HPLC analysis.

Analytical method. For the chromatographic analysis a Dionex Ultimate 3000 UPLC system (Thermo Scientific, San Jose, CA, EUA) equipped with a quaternary pump, an automatic injector (at 5ºC), a degasser and a column compartment with an automated thermostat was used. The detection of the compounds was carried out with a diode detector

(DAD) using the wavelengths of 280, 330 and 370 nm, coupled to a mass spectrometry (MS) detector.

For the separation of the compounds a Waters Spherisorb S3 ODS-2 C18 reverse phase column (4.6 × 150 mm, 3 μm; Milford, EUA) thermostated at 35°C was employed. The mobile phase used was formic acid/water (A, 0.1%) and acetonitrile (B). The elution gradient established was isocratic: 10% to 15% B up to 5 min, 15-20% B up to 5 min, 20-25% B 10 min, 25-35% B 10 min, 35-50% B 10 min and rebalancing the column for 10 min. A flow rate of 0.5 mL/min was defined. The HPLC system described was also connected to a mass spectrometer (MS).

The detection in the MS was performed using an Ion Trap Linear LTQ XL mass spectrometer (ThermoFinnigan, San Jose, CA, EUA), equipped with an ESI source (electrospray ionization source). Nitrogen (50 psi) was used carrier gas. The system worked with a spray voltage of 5 kV, an initial temperature of 325°C and capillary voltage of -20 V. The voltage of the tube lens offset was maintained at -66 V. The spectra were recorded in negative ion mode between 100 and 1500 m/z. The collision energy used was 35 (arbitrary units). Data were collected and analyzed using the Xcalibur® program (ThermoFinnigan, San Jose, CA, EUA).

For compounds identification, the obtained data (retention times, UV-Vis spectra and mass spectra) were compared with literature and, when available, with standards. Calibration curves for quantitative analysis were obtained by injection of standard solutions with known concentrations (2.5-100 μg/mL): quercetin-3-*O*-glucoside ($y= 34843x – 160173$; $R^2= 0.9993$), based on UV-Vis signals and using the maximum absorption wavelength of each standard compound. In the cases where there was no availability of standards for the respective compounds, the quantification was done through calibration curves for compounds of the same phenolic group. The results were expressed in mg of compound per g of extract.

3.5.4.2. Anthocyanin phenolic compounds

Extraction procedure. The lyophilized samples of the fruits (1 g) were subjected to a maceration at room temperature with magnetic stirring during 1h, employing a solution of EtOH/H_2O (80:20, *v/v*; 30 mL) acidified with citric acid (0.5%). Then, the mixture was filtered through filter paper (Whatman Nº4) and the remaining residue was re-extracted with

the extraction solution described above. In order to eliminate ethanol, the filtrate was evaporated at 35ºC (rotary evaporator, Büchi R-210, Flawil, Switzerland) and the remaining aqueous fraction was lyophilized (-47ºC, 0.045 bar; FreeZone 4.5, Labconco, Kansas City, MO, EUA). The obtained extract was subsequently re-dissolved in an EtOH/H_2O solution (80:20, *v/v*; 1 mL) and then filtered (0.22 µm LC disposable discs) for further analysis on a HPLC system.

Analytical method. Anthocyanins were analyzed using a high-performance liquid chromatography system (HPLC, Dionex UltiMate 3000 UPLC, Thermo Scientific) described in ***section 3.5.4.1***. For the separation of the compounds, an AQUA® C18 reverse phase column (5 µm, 150 mm x 4.6 mm id, Phenomenex) thermostated at 35 ºC was employed. The solvents used were: (A) 0.1% TFA in water and (B) 100% acetonitrile. The applied gradient was: 10% B up to 3 min, 10 to 15% B 12 min, 15% B 5 min, 15 to 18% B 5 min, 18 to 30% B 20 min and 30 to 35% 5 min, at a flow rate of 0.5 mL/min. The solvents used were 0.1% trifluoroacetic acid (ATF) in water (A) and acetonitrile (B). Gradient elution followed the following parameters: 10% B for 3 min, 10 to 15% B for 12 min, 15% B for 5 min, 15 to 18% B for 10 min, 18 to 30% B for 20 min, 30 to 35% B for 5 min and 35 to 10% B for 10 min. The total analysis time was 60 minutes, followed by reconditioning the column for 10 min, at a flow rate of 0.5 mL/min. The detection was performed with a DAD detector, using 520 nm as the main wavelength, and with mass spectroscopy connected in serie through the output of the DAD cell. MS detection was performed in positive mode using a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan) equipped with an ESI source. Nitrogen (50 psi) was used as a coating gas. The system was operated with a spray voltage of 4.8 kV, a source temperature of 320°C and a capillary voltage of 14 V. The tube lens shift was maintained at a voltage of 75 V. The sweep covered a mass amplitude of *m/z* 100 to 1500. The collision energy used was 20 (arbitrary units). Data acquisition was performed with the Xcalibur® data system (Thermo Finnigan).

The characterization of anthocyanins present in the samples was obtained using its UV-Vis and mass spectrum, with their retention times being compared with standards, when available. For quantitative analysis, calibration curves were obtained by injection of standard solutions with known concentrations (0.25-50 µg/mL): cyanidin-3-*O*-glucoside (y= 134578x

– $3x10^6$; R^2= 0.9986), and pelargonidin-3-*O*-glucoside (y= 61493x - 628875; R^2= 0.9957). The results were expressed in mg per g of extract.

3.6. Evaluation of bioactive properties of the *Malpighia emarginata* DC. fruits, through *in vitro* tests

Preparation of hydroethanolic extract. The extraction was carried out as described previously in ***Section 3.5.4.1***. The lyophilized extracts were re-dissolved to a concentration of 10 mg/mL in ethanol/water (80:20, *v/v*) for the antioxidant activity assays (***Section 3.6.1***); in dimethyl sulfoxide (DMSO) (100 mg/mL) for analysis of antimicrobial activity (***Section 3.6.2.***); and in distilled water to a concentration of 8 mg/mL for cytotoxic evaluation (***Section 3.6.3***), hepatotoxicity (***Section 3.6.4***) and anti-inflammatory activities (***Section 3.6.5***).

3.6.1. Antioxidant activity

3.6.1.1. TBARS

Lipid peroxidation can be determined by the products of the oxidation that react with thiobarbituric acid (TBA) giving rise to pink compounds that are known as thiobarbituric acid reactive species (TBARS). One of the products commonly used as a biomarker of lipid peroxidation is malodialdehyde (MDA). When it is associated with TBA, in the presence of H^+ ions, forms a chromogen (MDA-TBA) according to the reaction shown in **Figure 13**.

The methodology consisted in the oxidation of a lipid-rich preparation induced by a metallic ion (iron or copper) addition. The extension of the reaction was determined by the ability of the antioxidants present in the sample to stop the oxidation process with thiobarbituric acid, thus the chromogen formation is inhibited (less pink) (Gutteridge, 1995; Ng et al., 2000).

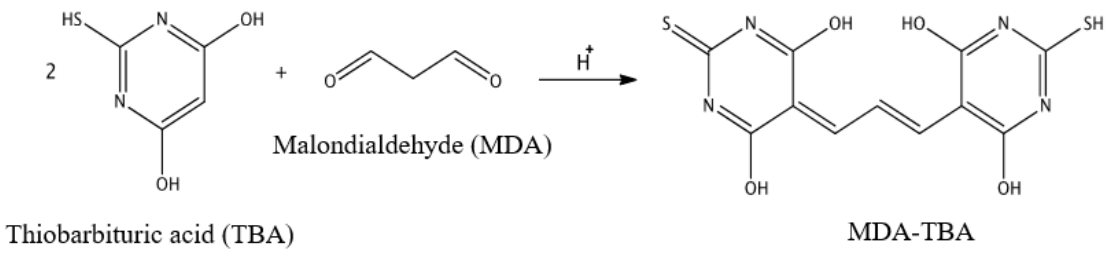


Figure 13. Formation of the complex MDA-TBA.

Porcine (*Sus scrofa*) brains were obtained from official slaughtered animals, dissected, and homogenised with Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4), to produce a 1:2 (*w/v*) brain tissue homogenate. It was centrifuged at 5175 *rpm* for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with different concentrations solutions (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% *w/v*, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, *w/v*, 0.38 mL), and the mixture was then heated at 80ºC for 20 min. After centrifugation at 3000 g for 10 min to remove the precipitated protein, the colour intensity of the MDA-TBA complex in the supernatant was measured by its absorbance at 532 nm (**Figure 14**). The inhibition ratio (%) was calculated using the **Equation 3**. Where: A is absorbance of the control and B, the absorbance of the compound solution.

$$Inhibition\ ratio(\%) = ((A - B))/A * 100$$

Equation 3. Equation of the inhibition ratio determination in TBARS assay.

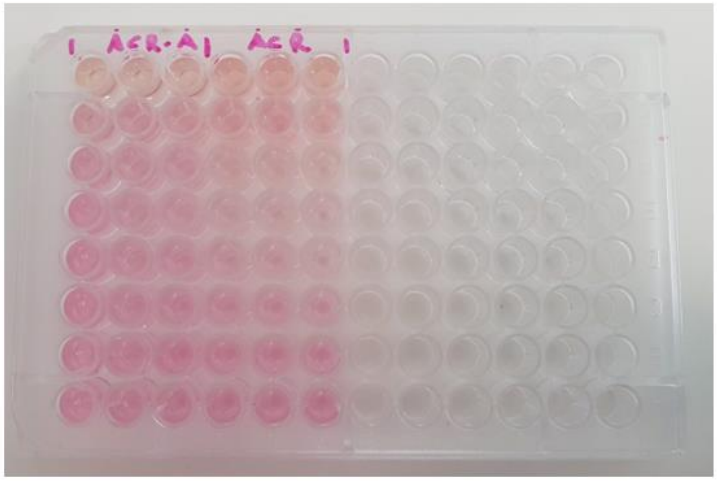


Figure 14. Evaluation of antioxidant activity by the TBARS method.
(Source: from the author).

3.6.1.2. OxHLIA

This method consisted in sheepd blood harvested. The blood sample was centrifuged (Multifuge X1R, Thermo Fisher Scientific; 2900 *rpm*, 5 min, 10°C) and the supernatant was discarded in order to recover only the erythrocytes. These were subjected to a first wash with NaCl (150 mM) followed by three washes with phosphate-saline buffer (PBS; pH 7.4), with centrifugation and removal of the supernatant at each wash (Evans et al., 2013). A solution

of erythrocytes at 2.8% was prepared (*v/v*), resuspending in PBS. In a 48-well microplate, 200 µL of the erythrocyte solution were added to: 400 µL of PBS (control), extract of the *M. emarginata* fruit dissolved in PBS (20 at 0.625 mg/mL) and water (to promote complete hemolysis). The microplates were preincubated with shaking (37°C, 10 min) for further addition of 200 µL of dihydrochloride of 2,2'-azobis(2-amidinopropan) (AAPH; 160 mM). After measuring the optical density at 690 nm, the microplates were incubated again under the same conditions, with measurements every 10 min (Takebayashi et al., 2012) (**Figure 15**). The percentage of the erythrocyte population that remained intact (PE) was calculated following the **Equation 4.**

$$PE\ (\%) = (S_t - \ 〚CH〛_0)/(S_0 - \ 〚CH〛_0) * 100$$

Equation 4. Percentage of the erythrocyte population (PE) in the OXHLIA assys.

S_t and S_0 correspond to the optical density of the sample at t and 0 min, respectively, and CH_0 is the optical density of complete hemolysis at 0 min. The results were expressed as hemolysis delay time (Δt), which was calculated following the **Equation 5**.

$$\Delta t\ (min) = \ 〚Ht〛_{50}\ (sample) - \ 〚Ht〛_{50}\ (control)$$

Equation 5. Hemolysis delay time.

Ht_{50} corresponds to the time (min) of the 50% of hemolysis, graphically obtained from the hemolysis curve for each concentration of antioxidant sample. Subsequently, linear correlations were established between the values of Δt and the different sample concentrations (Lockowandt et al., 2019). From these, the concentration capable of delaying hemolysis was calculated at 60 min (EC_{50} (60 min), mg/mL) and 120 min (EC_{50} (120 min), mg/mL).

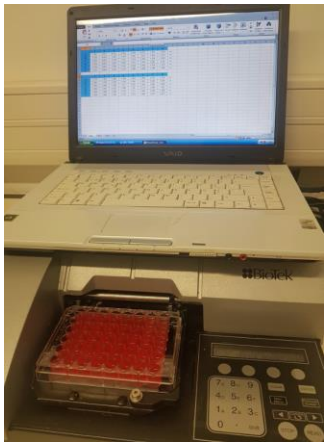


Figure 15. Evaluation of antioxidant activity by the OxHLIA method (Source: from the author).

3.6.2. Antimicrobial activity

3.6.2.1. Antibacterial activity

The methodology employed to evaluate antibacterial activity was previously described by Carocho et al. (2015). Gram-negative bacteria were used, such as: *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 35030) and *Salmonella* Typhimurium (ATCC 13311) and Gram-positive bacteria *Staphylococcus aureus* (ATCC 11632), *Bacillus cereus* (food isolated) *Listeria monocytogenes* (NCTC 7973). These microorganisms were purchased at the Mycological laboratory, Department of Plant Physiology, Institute for Biological research "Sinisa Stanković", University of Belgrade, Serbia. The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations, for each of the evaluated extracts, were determined using the microdilution method.

The bacterial cultures were adjusted through the spectrophotometer with a concentration of 1×10^5 CFU/mL, corresponding to a bacterial suspension determined in a spectrophotometer at 625 nm. The inoculum dilutions were grown in a solid medium to check the absence of contamination and to verify the inoculum validity. The different dilutions of the hydroethanolic extract were pipetted into the wells containing 100 µL of triptych soy broth (TSB) and, subsequently, 10 µL of inoculum were added. The microplates were incubated for 24 hours at 37ºC. For the determination of the MICs lower concentration that produced a significant inhibition (around 50%) of the growth of the bacterium in comparison

with the positive control. The MIC of the samples was detected following the addition of 40 µL, 0.2 mg/mL of iodonitrotetrazolium chloride (INT) and incubated at 37°C for 30 min.

The MICs obtained from the susceptibility test of various bacteria to the *M. emarginata* extract were also determined by a colorimetric microbial viability assay based on the reduction of the INT color and compared with a positive control for each bacterial strain. The MBC (minimum bactericidal concentration) was determined by subculture in series, placing 10 µL of each well that did not change color at 100 µL of TSB. The lowest concentration that did not show growth after this subculture was considered the MBC. Streptomycin and ampicillin were used as positive controls, while 5% dimethyl sulfoxide (DMSO) was used as a negative control (**Figure 16**). The results of MIC and MBC were expressed in mg/mL of lyophilized hydroethanolic extract.

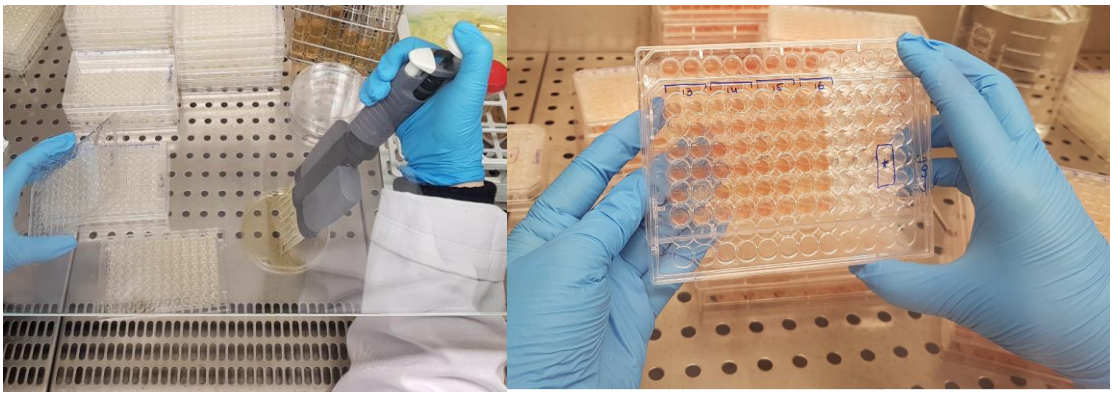


Figure 16. Evaluation of antibacterial activity by the microdilution method.
(Source: from the author).

3.6.2.2. Antifungal activity

For the evaluation of antifungal activity, the procedure previously described by Carocho et al. (2015) was employed. For this, *Aspergillus fumigatus* (ATCC 9197), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Penicillium funiculosum* (ATCC 36839), *Penicillium aurantiogriseum* (food isolate) or *Trichoderma viride* (IAM 5061) were used as the microfungi. These organisms were purchased at the Mycology Laboratory of the Department of Plant Physiology of the Biological Research Institute “Siniša Stanković” at the University of Belgrade in Serbia.

The micromycetes were kept on malt agar (MA) and the cultures were stored at 4°C and subcultured once a month. The fungal spores were washed from the surface of the agar plates with 0.85% sterile saline solution containing 0.1% (*v/v*) of Tween 80. The spore

suspension was adjusted with a sterile saline solution to a concentration of approximately 1.0×10^5 CFU/mL in a final volume of 100 μL per well. Inocula were stored at 4°C. The inoculum dilutions were grown in solid MA to verify the absence of contamination and the inoculum validity. The determination of the MIC was performed by the successive dilution technique in 96-well microplates. The sample was added to the malt medium with the fungal inoculum and the microplates were incubated for 72 hours at 28°C. The lowest concentrations without visible growth (using a binocular microscope) were defined as MIC. The minimum fungicidal concentrations (MFCs) were determined by subculture in series of 2 μL of each well that did not change colour, in microplates containing 100 μL of malt broth per well and later incubated for 72 h at 28°C. The lowest concentration without visible growth was defined as MFC, indicating 99.5% of death of the original inoculum. As a negative control 5% DMSO was used, while bifonazole and ketoconazole were used as positive ones.

The results of MIC and MFC were expressed in mg/mL of hydroethanolic extract.

3.6.3. Cytotoxic activity

The evaluation of the cytotoxic potential of the hydroethanolic extracts (80:20, *v/v*) of the *M. emarginata* fruits was performed using the Sulforodamine B (SRB) assay, previously described by Barros et al. (2013) (**Figure 17**).

To test the anti-proliferative ability of the extract in tumor cells, several human tumor cell lines were used, such as: MCF-7 (breast carcinoma), NCI-H460 (lung carcinoma), AGS (gastric carcinoma) and CaCo (intestinal carcinoma); obtained from DMSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. These lines were maintained as adherent cultures in RPMI-1640 medium supplemented with 10% FBS (MCF-7 and NCI-H460) or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (HeLa and HepG2 cells), at 37°C in an incubator with humidified air and 5% CO_2.

In order to avoid contamination, all the procedures were performed in an aseptic environment and in a laminar flow chamber (TLStar, AV-30/70). Concentrations from 0.125 to 8 mg/mL were tested by water dilution. The cells were maintained in RPMI-1640 medium containing 10% Fetal Bovine Serum (SFB) inactivated by heat (MCF-7 and NCI-H460) or in DMEM supplemented with 10% SFB, 2 mM glutamine, 100 U/mL penicillin and 100

μg/mL streptomycin (HeLa and HepG2 cells), in an incubator with established conditions of 37°C, humidified air and 5% CO_2 (HF 151, Heal Force).

First, the culture medium was removed from each box containing the cell lines and the washing medium (HBSS, 2 ml) was added. In order to cells detach, the proteolytic enzyme trypsin (1.5 ml) was added and the box was placed in the incubator for 3 minutes. After that, culture medium (3 mL) was added to inactivate trypsin, thereby preventing cell destruction. The cell suspension obtained was pipetted and transferred to a sterile falcon to centrifuge at 1200 *rpm* for 5 min at 10ºC (Centurion K24OR, West Sussex, United Kingdom).

The supernatant was then discarded and the pellet was suspended in 5 mL of culture medium. For cells counting, 50 μL of the suspension were removed and 50 μL of trypan blue solution were added. The mixture was homogenized and the counting was done in a Neubauer chamber. Each cell line was plated at an appropriate density (1.0×10^4 cells/well), according to the performed calculations, in 96-well plates. Six different sample dilutions of *M. emarginata* (10 μL, 400-6.25 μg/mL) were added in each well, together with the volume of cells mentioned above, making up the volume of each well with culture medium. The microplates were sealed and stored in the incubator for 24 hours until the SRB test. After the incubation period, cold trichloroacetic acid (TCA, 10%; 100 μL) was added to each well, then incubated for another 60 min at 4 ºC. The microplates were washed with deionized water and dried. Then, SRB solution (0.1% in 1% acetic acid; 100 μL) was added, leaving the plate to incubate for 30 min at room temperature.

Subsequently, the plate was washed with acetic acid (1%) to remove excess SRB and allowed to air dry. The SRB was solubilized with 200 μL of Tris (10 mM; pH 7.4) on a microplate shaker (Stat Fax-2100). Absorbance was measured at 540 nm in a microplate reader (Bio-Tek Instruments, ELX800, Inc; Winooski, EUA), using ellipticin as a positive control. The results were expressed in GI_{50} values (sample concentration that inhibits 50% of cell growth) in units of μg/mL.

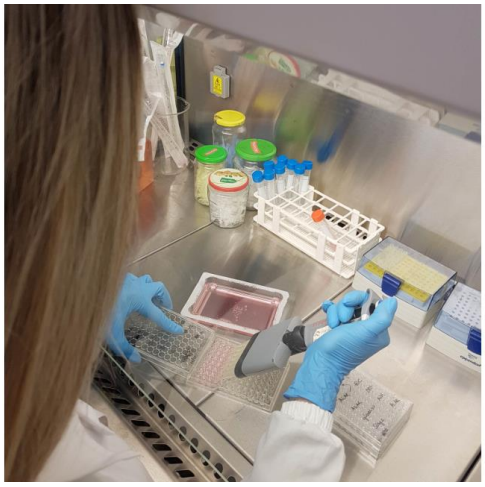


Figure 17. Evaluation of citotoxicity by the Sulforodamine B (SRB) assay.
(Source: from the author).

3.6.4. Assessment of citotoxicity in non-tumor cell line

A culture of non-tumor primary cells PLP2 were used for the hepatotoxicity assay (**Figure 18**). The PLP2 cells were obtained through pig liver cells. The liver tissue was washed with Hank's solution containing 100 U/ml of penicillin and 100 µg/ml of streptomycin. It was further divided into explants of approximately 1 mm^3. Some of these explants were placed in 25 cm^2 flasks containing DMEM medium supplemented with FBS (10%), non-essential amino acids (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL). The flasks were incubated at 37 ºC with humidified atmosphere and 5% CO_2. The medium was replaced every two days of incubation and cell growth was monitored by observation with a phase contrast microscope.

A sample (190 µL) of the cell culture with adequate density ($1.0x10^4$ cells/well) was transferred to 96-well microplates with 10 µL of the different dilutions of the extract, mentioned in ***Section 3.6.3.*** (400 to 6.25 µg/mL). Then it was incubated at 37ºC for 48 hours. The SRB test previously described (***Section 3.6.3.***) was performed. Ellipticin was used as a positive control and the results were expressed as GI_{50} (sample concentration that inhibits 50% cell growth in PLP2 cell culture) in µg/mL units (Barros et al., 2013).

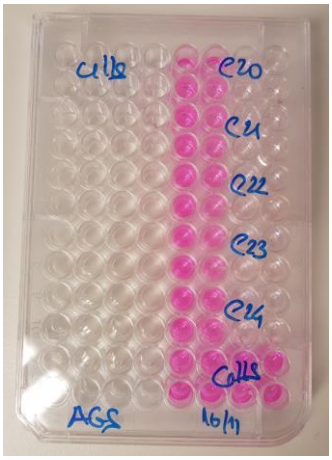


Figure 18. Representative image of microplate for cytotoxicity measurement. (Source: from the author).

3.6.5. Anti-inflammatory activity

For the evaluation of anti-inflammatory activity macrophage cells RAW 264.7 were used, according to Jabeur et al. (2016) protocol. Cell cultures were made in DMEM medium, supplemented with 10% heat-inactivated bovine serum and L-glutamine, at 37°C with 5% CO_2, in humidified air.

The cells with active growth were released with a cell scraper. The experimental cell density was established at 5×10^5 cells/mL and the proportion of dead cells was less than 1%, according to the Trypan Blue exclusion test. Then, the cells were distributed in a 96-well plate (150,000 cells/well) and allowed to adhere to the microplate overnight.

Subsequently, the cells were treated with different concentrations of the extract of the fruit of *M. emarginata* fruits for 1 hour, followed by stimulations with lipopolysaccharides (LPS) (1 µg/mL) over 18 hours. Controls were prepared without the addition of LPS in order to see if they induced changes in baseline levels of nitric oxide (NO).

The presence of nitric oxide was determined using a Griess Reagent Kit (Promega) containing sulfanilamide, N-(1-naphthyl) ethylenediamine hydrochloride (NED) and nitrated solutions. The cell supernatant (100 µL) was transferred to the plate and mixed with sulfanilamide and NED solution, 5 to 10 minutes each, at room temperature. The produced nitric oxide was determined by measuring the absorbance at 540 nm (ELX800 Biotek microplate reader) and compared to the calibration curve (Jabeur et al., 2016).

3.7. Optimization of the extraction process to obtain a natural dye based on anthocyanins, from the fruit of *Malpighia emarginata* DC.

3.7.1. Experimental design for extraction optimization

A central composite rotatable design (CCRD) combining five-levels of the independent variables X1 (time, *t*, 2–90 min), X2 (temperature, *T*, 20–90ºC) and X3 (solvent, 0–100% ethanol/water, *v/v*) was implemented to optimize the extraction of anthocyanins from *M. emarginata* bioresidues using RSM (**Table 7**). These variables and the respective range of values were selected based on previous optimization studies (Albuquerque et al., 2020; Rocha et al., 2020). The Design-Expert software, Version 11 (Stat-Ease, Inc., Minneapolis, USA) was used to generate the 20 experimental points of the CCRD design by entering the factor ranges in terms of alphas ($\alpha = 1.68$). The design included 8 factorial points, 6 axial or star points chosen to allow rotatability, and 6 replicated centre points. The 20 runs were randomized to minimize the effects of unexpected variability.

Table 7. Natural and codded values of the independent variables used in the five-level central composite rotatable design (CCRD) used to optimize the extract.ion of anthocyanins from *M. emarginata* bioresidues.

CODED VALUES	**NATURAL VALUES**		
	Time (min)	*Temperature (W)*	*Solvent (ethanol %, v/v)*
-1.68	2	20	0
-1	20	34	20
0	46	55	50
+1	72	76	80
+1.68	90	90	100

3.7.2. A heat assisted extraction (HAE)

Heat-assisted extraction (HAE) consists of extracting molecules from a powdered sample using an acidified solvent under defined conditions of temperature, time and agitation (Wang et al., 2016). Considering the simplicity and the reduced number of equipments of this technique, it seems to be ideal for industrial purposes (Roriz et al., 2017; Backes et al., 2018; López et al., 2018).

The extraction procedure (**Figure 19**) was carried out in a water bath using a magnetic stirrer (CimarecTM, Thermo Scientific) under a fixed speed (5000 *rpm*). Closed bottles were

used in order to avoid solvent evaporation. Solvent (20 mL, ethanol/water) acidified with 0.05% of citric acid (pH ≈ 3) was added to 1 g of powdered sample (*M. emarginata* fuit) for the extraction. This procedure was performed following the extraction conditions predefined by the established RSM model explained in ***section 3.7.1***.

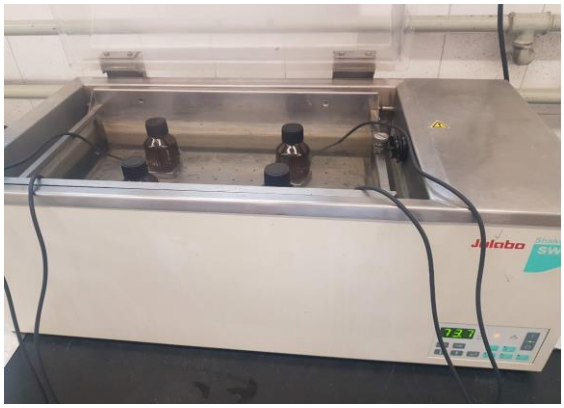


Figure 19. HAE extraction process of the anthocyanins from *Malpighia emarginata* DC. fruits. (Source: from the author).

3.7.3. Preparation of extracts obtained by HAE

Subsequent to the extraction process, the extract solutions were centrifuged (5000 *rpm*, for 10 minutes at 10ºC) and filtered through filter paper (Whatman N°4), in order to remove suspended solids. The supernatants were collected and divided into two fractions: one for HPLC-DAD analysis (**Figure 20A**) and the second for determination of extraction yield. The separate fraction for HPLC analysis (1.5 mL) was filtered through LC syringe filter (0.22 μm) and then injected. The second fraction was collected to determine the extraction yield (5 mL) (**Figure 20B**), followed by a drying process at a temperature of 105ºC for 48 hours, for subsequent weighing of the solids (**Figure 20C**).

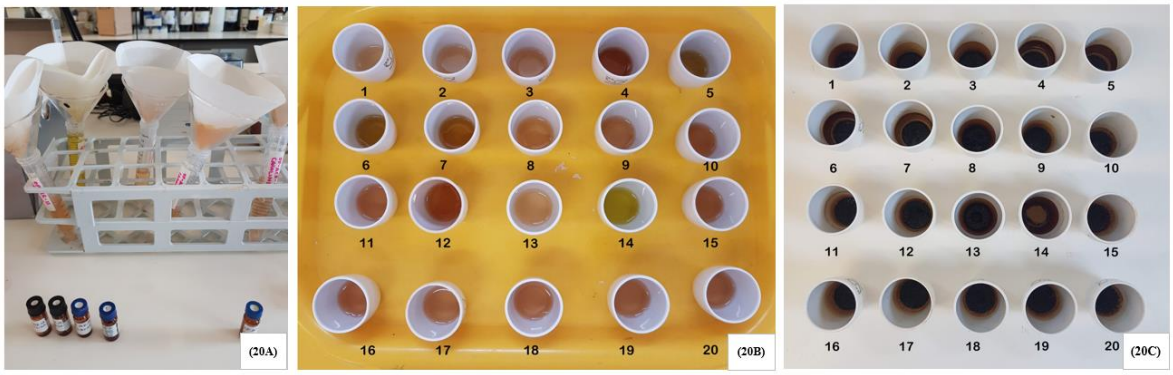


Figure 20. The obtained extracts for HPLC-DAD analysis (A), for determination the extraction yield: before drying (B) and after drying (C).
(Source: from the author).

3.7.4. Extraction Yield

The extraction yields (%) were calculated based on the dry weight (crude extract) obtained after evaporation. To further dissolve the extract, 5 mL of acidified water (0.05% citric acid) were added. The obtained solution was filtrated by a LC filter disk (0.22 µm).

In all cases, the filtrates were concentrated at 35°C in a rotary evaporator (Büchi R-210, Flawil, Switzerland) under reduced pressure and the aqueous phase was then freeze dried to obtain a dried extract.

3.7.5. Identification and quantification of anthocyanin compounds through an HPLC-DAD-ESI/MS system

The analysis of the colourant extracts rich in anthocyanin compounds, obtained by the HAE method, was performed by a Dionex Ultimate 3000 HPLC system (Thermo Scientific) coupled to a DAD and mass spectrometry detector, previously described in ***section 3.5.4.1***.

3.7.6. Extraction process modelling and statistical analysis

The dependent variables Y_1 (extraction yield, %, *w/w*), Y_2 (cyaninidin-*O*-deoxyhexoside content – COD, mg/g extract), Y_3 (pelargonidin-*O*-deoxyhxoside content – POD, mg/g extract), and Y_4 (content of both anthocyanins – total, mg/g) were used in the optimization of the extraction process. The response surface models were fitted by means of least squares calculation using the following second-order polynomial equation (**Equation 6**):

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2$$

Equation 6. Second-order polynomial equation.

Where Y corresponds to the dependent variable to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of the linear effect, b_{ij} is the coefficient of the interaction effect, b_{ii} is the coefficient of the quadratic effect and n is the number of variables.

Fitting procedures, coefficient estimates and statistical analysis were performed using Design-Expert software. The analysis of variance (ANOVA) was used to assess the significance of the model generated and of all the terms that make up the model, as well as the lack-of-fit. The method for testing statistical significance was performed by calculating the p-value from the F-value, considering the existence of significance for $p < 0.05$. Only the statistically significant terms were used in construction of the theoretical model. Coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}) and adequate precision were used to estimate the adequacy of the polynomial equation to the response. The lack-of-fit measures the quality of the model's fit to the experimental data. Thus, it must be non-significant ($p > 0.05$).

3.8. Statistical analysis

The tests mentioned in this study were performed in triplicate and the results were expressed as mean ± standard deviation (SD). The statistical analysis of the data was carried out in order to determine the significant differences between the samples. It was carried out through an analysis of variances one-way ANOVA and the t-student test, considering the different types of comparisons. In each table, the statistical test applied was described. For this, the SPSS v program was used. 23.0 (IBM Corp., Armonk, New York, EUA).

Results and Discussion

4. Results and discussion

4.1. Colour of *Malpighia emarginata* DC. fruits

The color is a very important parameter in the food industry, since the visual aspect of a product generates an initial impact on the consumer, which is sometimes the decisive factor for its acceptance or not (Lin et al., 2018).

The results of the chromatic analysis in the colour space CIE *L*a*b** of the *M. emarginata* fruits are presented in **Table 8**. The luminosity scale (*L**) ranged between 0 and 100, and the *a** (from green to red) and *b** (from blue to yellow) parameters ranged between -120 and 120 (Xu et al., 2016). The colour of the freeze-drying fruit for the *L** parameter revealed, on average, a value of 40.1 ± 0.9 and *a** and *b** parameters showed values of 32.7 ± 1.4 and 15.2 ± 0.3, respetively. Otherwise, in crushed fruit the *L** parameter evidenced a value of 59.3 ± 1.2, but in the *a** and *b** parameters some differences were noticed, showing values of 16.5 ± 0.3 and 29.6 ± 0.3, respectively.

For better understanding the colour shape, the values obtained were converted to RGB values through a program (http://www.easyrgb.com/en/convert.php) and the different types of colouring are presented in **Table 8**.

Table 8. The colour measurement of the liofilized *Malpighia emarginata* DC. fruits.

SAMPLES	*L**	*a**	*b**	RGB colour
Whole fruits	40.1 ± 0.9	32.7 ± 1.4	15.2 ± 0.3	
Crushed fruits	59.3 ± 1.2	16.5 ± 0.3	29.6 ± 0.3	
p-value	0,182	0,198	0,193	

Other studies were carried out over *M. emarginata*, in order to determine colour parameters. Canuto et al. (2010) studied the physico-chemical characterization of *M. emarginata* from Amazonas, and the colour was determinated using a Konica Minolta colorimeter model CR-400 (Osaka, Japan) operating on the CIELAB scale (*L**, *a** and *b**). The evaluation was done in the preparation of fruit pulps (fresh samples) and the values obtained were 23.8 for the parameter *L**, 16.401 for the coordinate *a**, and 10.732 for the coordinate *b**.

Delva and Schneider (2013a) also evaluated the colour of the *M. emarginata* mature fruit, but in this case, they used a machine vision system which was composed of a light box and a Nikon D200 digital colour camera coupled with a computer having a fire wire connection. A computer program collected images and obtained colour results based on lightness (*L**), redness (*a**) and yellowness (*b**) values of the fruits. With this system the values were 43.73 for the parameter *L**, 38.75 for the coordinate *a**, and 31.37 for the coordinate *b**.

The found differences could be attributed to the variation of the fruit matrix and the technique employed in each case.

4.2. Nutritional value of the *Malpighia emarginata* DC. fruits

The results of the evaluation of the nutritional value of *M. emarginata* fruits are shown in **Table 9**. Considering the obtained results, carbohydrates (8.57 ± 0.04 g/100 g fw) were the macronutrients presented in higher amounts, followed by proteins (1.15 ± 0.04 g/100 g fw). The fat concentration was considerably low, presenting a value of 0.30 ± 0.001 g/100 g fw. In terms of moisture, 89.74 ± 0 g/100 g was determined, showing a high-water content in its composition. The energy level of the *M. emarginata* fruits was 39.42 ± 0.04 kcal/100 g fw.

Table 9. Nutritional parameters of *Malpighia emarginata* DC. fruits (mean±SD).

	CONCENTRATION
Moisture (g/100 g fw)	89.74 ± 0
Ash (g/100 g fw)	0.51 ± 0.01
Proteins (g/100 g fw)	1.15 ± 0.04
Fat (g/100 g fw)	0.06 ± 0.001
Carbohydrates (g/100 g fw)	8.57 ± 0.04
Energy (kcal/100 g fw)	39.42 ± 0.04
Energy (kJ/100 g fw)	165.04 ± 0.20

fw- fresh weight

Monteiro et al. (2020) evaluated the nutritional value of bagasse and flour obtained from the acerola (*Malpighia punicifolia*) agroindustrial residue with potential use as fiber source. This study presented high carbohydrates concentration (10.8 g/100 g fw), followed

by proteins (1.4 g/100 g fw) and, finally, fats (1 g/100 g fw). The lowest concentrations were detected in ash and moisture contents, with values of 0.3 and 86.5 g/100 g fw, respectively. As can be seen, small differences were observed comparing to the present study, which may be probably due to the differences in the methodologies used to obtain the samples, or the origins of the raw material.

There is not enough information in the cientific literature about *M. emarginata* characterization. Nevertheless, in a study performed by Aguiar et al. (2010), the nutritional value of *Malpighia punicifolia* Linn seeds, previously dried and processed into flour, were analyzed. In this work, high carbohydrates concentration was detected (57.24 ± 2.44 g/100 g fw), followed by proteins (16.94 ± 0.8 g/100 g fw) and fats (3.92 ± 0.02 g/100 g fw). The lowest concentration was detected in ash contents (0.44 ± 0.01 g/100 g fw). Comparing these values with those presented in **Table 10**, it is possible to observe that the nutriotinal profile is higher in the seeds that in the fruit itself. This observation could be explained since the seeds contain the embryo, as well as its nutrients.

In comparison with the studies carried out by Jahan et al. (2011), where they determined the nutritional profile of some tropical fruits, the value obtained for the energetic value in the present work is intermediate. These authors obtained results of 16.85 kcal for melon, 33.14 kcal for golden apple, 46.66 kcal for mango and 53.88 kcal for blackberry.

4.3. Chemical composition of the *Malpighia emarginata* DC. fruits

4.3.1. Free sugars, organic acids and fatty acids

The results of the chemical composition (free sugars, organic acids and fatty acids) of *M. emarginata* fruits are presented in **Table 10**.

Regarding the sugar profile, the samples showed the presence of three sugars groups, two monosaccharides (glucose and fructose) and one disaccharide (sucrose). Fructose was the main free sugar detected (1.14 g/100 g fw), while sucrose was the molecule identified at the lowest concentration (0.15 g/100 g fw). Glucose was also detected with a value of 0.89 g/100 g fw. The total sugar content was 2.2 g/100 g fw.

Vendramini and Trugo (2000), investigated the chemical composition of *Malpighia punicifolia* L. considering the effect of the stage of maturity. The concentration of sugars increased with the progress of maturation, being 4.4 g/100 g at mature stage. The value

obtained by these authors is almost double that of the present investigation, this difference is probably because the fruit is more mature.

Moura et al. (2018) also studied the total sugars concentration as function of the maturity stages. The authors found that total sugar concentration varied between 2.75 and 6.03 %. They had concluded that concentration increased with the maturing stage of the fruit.

The organic acids were also evaluated in this study and the results are presented in **Table 10**. Oxalic, malic, ascorbic and succinic acids were detected in *M. emarginata* samples. The most abundant compound (0.75 ± 0.02 mg/100 g fw) was identified as malic acid, followed by ascorbic acid (0.554 ± 0.003 mg/100 g fw) and oxalic acid (0.099 ± 0.002 mg/100 g fw). The total concentration of organic acids was 1.403 ± 0.030 mg/100 g fw.

Malic acid has been widely used in different sectors, such as in the food and beverage industries (used as a flavour enhancer and acidulant), chemicals, pharmaceuticals and also in agriculture (Zhe et al., 2014). In addition, therapeutically, malic acid consumption may increase urine pH and citrate levels, making stone formation less likely. The authors concluded that malic acid supplementation may be useful for the conservative treatment of calcium kidney stones (Rodgers et al., 2014).

Ascorbic acid (most commonly known as vitamin C) is an essential vitamin in the human body. This compound interfer in the proper formation and maintenance of intercellular material. It is also involved in the development of connective tissues, lipid and vitamin metabolism, hormone and neurotransmitter synthesis, immune function, and wound healing. As a powerful antioxidant and reducing agent, it acts in the free radical detoxification process (Latham, FAO, 2002; Bastias and Cepero, 2016).

Different groups of researchers have determined the ascorbic acid content in agro-industrial residues of *M. emarginata*.

Sancho et. al (2015) studied the physicochemical parameters (pH, total soluble solids, water activity, reducing sugar, acidity, protein, moisture, ash, and lipids), functional compounds (total phenolic content, anthocyanins, ascorbic acid, and free radical scavenging activity—DPPH), fatty acid profile, and mineral content of seven fruit wastes, using titration with standardized 2,6-dichlorophenolindophenol sodium to determinate ascorbic acid. The obtained results of ascorbic acid showed a value of 170.73 ± 0.46 mg/100 g in the edible portion of the fruit.

Scherer et al. (2008), determined the presence of organic acids in *M. emarginata* pulp using a liquid chromatograph. The most predominant compound was ascorbic acid with a value of 494.07 ± 65.95 mg/100 g fw, followed by malic acid (344.54 ± 55.30 mg/100 g fw). The considerable differences among these values and those presented in **Table 10**, shoud be assigned to the evaluated substrate. While Scherer and co-workers studied the pulp, in this protocol the whole fruit was evaluated.

Moura et al. (2018) evaluated the chemical composition and antioxidant activity of juice from immature and mature *M. emarginata* fruits, using a HPLC system equipped with a UV detector. The results showed a higher concentration of malic acid, with a percentage fruits between 0.25% and 0.38%, followed by citric (0.01% - 0.03%) and tartaric acid (0.002% - 0.01%), for the percentage of total acids in mature fruits.

Silva et al. (2019), studied the acerola residues using titration, through a method based on the reduction of 2,6-dichlorophenol-indophenol by ascorbic acid. The results reported 134.6 ± 1.1 mg/100 g of dry residue of ascorbic acid.

Finally, the chemical evaluation also included the determination of fatty acid profile, and the results are shown in **Table 10**. Fourteen fatty acid were identified in the *M. emarginata* fruits, with oleic acid (C18:1n9c) and palmitic acid (C16:0) detected in the highest percentage (31.830 ± 0.677 % and 24.44 ± 0.16 %, respectively). In lower percentages the acids: linoleic (C18:2n6c; 17.4 ± 0.5 %), α-linoleic (C18:3n3; 10.8 ± 0.4 %), stearic (C18:0; 7.9 ± 0.1%), lignoceric (C24:0; 3.0 ± 0.1%) and myristic (C14:0; 1.27 ± 0.02%) were detected. The remaining identified fatty acids had concentrations below 1%.

It has been proved that palmitic and oleic acid, which are common in fruits seeds and peels, exert atheroprotective effects. They mainly act through lowering the total and low-density lipoprotein (LDL) cholesterol or through a decrease in coronary risk factors, such as diabetes or obesity (WHO, 2003). Particullarly, oleic acid consumption benefits serum, total lipid, riacylglycerol and glutathione levels, decreased tissue factor activities of brain and kidney in diabetic-hyperlipidemic and have protective effects against cardiovascular complications of diabetes (Emekli-Alturfan et al., 2010).

The saturated fatty acids (SFA; 39.4 ± 0.2 %) were the main group of fatty acids present in the *M. emarginata* extract. However, monounsaturated fatty acids (MUFA; 32.4 ± 0.7 %) and polyunsaturated fatty acids (PUFA; 28.2 ± 0.9 %) were quite close in concentrations.

According to Morales-de la Peña et al. (2011) the consumption of monounsaturated fatty acids (MUFA), especially oleic acid, has shown to decrease plasma triacylglycerol, cholesterol concentrations in healthy normolipidemic subjects and improve insulin sensitivity.

Also, polyunsaturated fatty acids (PUFA) are essential molecules for human health, playing an important role in blood-brain barrier functions, neural membrane fluidity, neurogenesis, memory, emotions and in the development and maintenance of brain structure during embryonic and adult stages (Khan and He, 2017, Yang et al., 2018). The consumption of these compounds decreases the risk of coronary heart disease and lowers LDL (Low density lipoprotein) cholesterol concentration and the total cholesterol/HDL (high density lipoprotein) cholesterol ratio (FAO, 2010). Studies report that insufficient intake of polyunsaturated fatty acids increases the probability of age-related diseases such as cardiovascular, brain, inflammatory and cancer (Kraic et al., 2018).

Other studies have been developed in order to evaluate the fatty acid profile of *M. emarginata*. Sancho et al. (2015) studied the characterization of the industrial residues of seven fruits, among them the *M. emarginata* fruits. In this research work, some differences can be observed between the values obtained in some fatty acids compared with the results of the present study. Authors observed a highest presence of linoleic acid (C18:2n6c; 32.2%), followed by palmitic (C16:6; 27.7%), oleic (C18:1n9; 23.2%) and stearic acid (C18:0; 14.2%). In smaller quantities, were detected the palmitoleic (C16:1) and myristic acid (C14.0) with values of 1.4 and 1.1%, respectively. Differences between those results and the presented in **Table 10** could be assigned to the type of sample used, e.g. pulp, peel, or whole fruit, the sample treatments used, or even the maturity stage of the sample.

Table 10. Composition of sugars, organic acids and fatty acids in the fruits of *Malpighia emarginata* DC. (mean±SD).

FREE SUGARS (g/100 g fw)		**FATTY ACIDS (%)**	
Fructose	1.14 ± 0.04	C12:0	0.26 ± 0.01
Glucose	0.89 ± 0.02	C13:0	0.110 ± 0.004
Sucrose	0.15 ± 0.01	C14:0	1.27 ± 0.02
Total	2.18 ± 0.06	C15:0	0.199 ± 0.004
		C16:0	24.44 ± 0.16
		C16:1	0.54 ± 0.02
		C17:0	0.49 ± 0.01
ORGANIC ACIDS (mg/100 g fw)		C18:0	7.9 ± 0.1
Oxalic acid	0.099 ± 0.002	C18:1n9	31.830 ± 0.677
Malic acid	0.75 ± 0.02	C18:2n6	17.4 ± 0.5
Ascorbic acid	0.554 ± 0.003	C18:3n3	10.8 ± 0.4
Succinic acid	tr	C20:0	0.89 ± 0.04
Total	1.403 ± 0.030	C22:0	0.81 ± 0.01
		C24:0	3.0 ± 0.1
		SFA	39.4 ± 0.2
		MUFA	32.4 ± 0.7
		PUFA	28.2 ± 0.9

tr: traces. Lauric acid (C12:0); Tridecyl acid (C13:0); Myristic acid (C14:0); Pentadecylic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Margaric acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linolenic acid (C18:2n6c); α-linolenic acid (C18:3n3); Arachidic acid (C20:0); Behenic acid (C22:0); Lignoceric acid (C24:0). SFA – Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA - Polyunsaturated fatty acids s.

Regarding the chemical composition, some comparison studies revealed significant differences. This can be explained due to several factors, namely by the application of different extraction methods and solvents, the different cultivation conditions of this plant species, which reflects a high influence on the chemical composition of the plants, and maturation stage of the fruit.

4.3.2. Phenolic compounds: non-anthocyanin and anthocyanin compounds

The detailed profile of phenolic compounds presents in the samples of *M. emarginata* fruit is listed in **Table 11**. The attempted identification of the compounds was based on retention times (Tr), wavelengths of maximum absorption in the UV-Vis region (max), pseudomolecular ion ($[M-H]^-$; $[M-H]^+$) and molecular ion fragmentation (MS^2), and, when possible, comparing with available standards or literature.

A total of eight compounds were identified (**Figure 21**), six non-anthocyanin and two anthocyanin molecules. All the non-anthocyanin compounds identified are flavonoids,

namely quercetin, kaempherol and isorhamnetin derivatives. Regarding the anthocyanin compounds, cyanidin glycosylated derivatives were identified and also a compound derived from pelargonidin.

Regarding the non-anthocyanin flavonoid derivatives, compounds 1 and 2 ($[M-H]^-$ at *m/z* 431), were identified as kaempherol-*O*-deoxy-hexoside, based on their UV spectra (λmax about 330 nm) and the yield of the MS^2 fragment at *m/z* 285(100). Peaks 3 and 4 were identified as quercetin-*O*-pentosyl-hexoside, with $[M-H]^-$ at *m/z* 595, based on their UV spectra (λmax about 347 and 348 nm) and the yield of the MS^2 fragment at *m/z* 301(100). Peaks 5 was identified as isorhamnetin-*O*-dipentosyl-hexoside, with $[M-H]^-$ at *m/z* 741, based on their UV spectra (λmax about 350) and the yield of the MS^2 fragment at *m/z* 315(100). Peaks 6 was identified as isorhamnetin-*O*-pentosyl-hexoside, with $[M-H]^-$ at *m/z* 609, based on their UV spectra (λmax about 340) and the yield of the MS^2 fragment at *m/z* 315(100).

The concentration obtained for all non-anthocyanin compounds was similar, isorhamnetin-*O*-pentosyl-hexoside was the main one, with values of 0.192 ± 0.0004 mg/g of extract. The remaining compounds exhibited concentrations between 0.190 ± 0.0002 and 0.185 ± 0.0001 mg/g of extract.

A study performed by Settu and Manju (2017) indicates that isorhametin compound has multiple bioactive properties, including antimicrobial, antioxidant, anticancer, neurological, cardiovascular, hepatoprotective, anti inflammatory and protective of the reproductive system. Especially in an *in vivo* study, it has been reported that this molecule has antioxidative enzyme activities on the concentrations of cholesterol and lipoperoxide in the serum and liver. In another case of study, performed by Kim et al. (2019), it was demonstrated that isorhamnetin exerted potent anti-inflammatory effects on BV2 microglial cells (a brain microglial cell line).

Similarly, this compound has antiproliferation effects against lung cancer cell lines (A549 cells) in *in vivo* and *in vitro* studies. These antiproliferative, apoptotic, necrotic and cell cycle effects suggest that isorhamnetin may have clinically significant therapeutic and chemopreventive capabilities (Settu and Manju; 2017).

The anthocyanin compounds identified represent 79.6% of the total phenolic compounds. Compound 1 was the majority anthocyanin compound. It was identified as cyanidin-*O*-deoxyhexoside ($[M-H]^+$ at *m/z* 433), based on its UV spectra (λmax about 330

nm) and the yield of the MS^2 fragment at *m/z* 287(100). The compound 2 ($[M-H]^+$ at *m/z* 417) appeared in lower concentrations and was identified as pelargonidin-*O*-deoxyhexoside, based on its UV spectra (λmax about 330 nm) and the yield of the MS^2 fragment at *m/z* 271(100). The values obtained were 3.1±0.1 and 1.37±0.05 mg/g of extract for each of them, respectively.

The phenolic compounds profile of *M. emarginats* fruits was previously characterised by several authors, such as Hanamura et al. (2005). These authors identified the structures of acerola polyphenols and elucidated their functional properties, extracting with methanol and trifluoroacetic acid, and they identified three polyphenolic compounds: cyanidin-3-*α*-Orhamnoside, pelargonidin-3-*α*-Orhamnoside and quercetin-3-*α*-Orhamnoside. However, the results obtained by these authors showed a phenolic profile very different. It could be explained by the different geographical origin of the samples and the different extraction conditions applied, including the solvent used.

According to Giuliani et al. (2016), cyanidin and pelargonidin are most common in nature. These compounds are present mostly in pigmented leaves, followed by fruits and flowers. In the study conducted by Almeida and Azevedo (2007), cyanidin was found to have the highest antioxidant efficiency relative to pelargonidin. They also concluded that both compounds possess the ability to decrease the susceptibility of human LDL to oxidative modifications induced by some azocompounds. These authors explain in their work that one of the reasons for the greater antioxidant activity of cyanidin compared to pelargonin is that the catechol structure in the B-ring of cyanidin confers high stability to phenoxyl radicals by participating in electronic delocalization.

Table 11. Retention time (Tr), wavelengths of maximum absorption in the UV-Vis region (λ_{max}), tentative identification and quantification of phenolic compounds in the hydroethanolic extract of *M. ermaginata* (mean±SD).

PEAK	Tr (min)	λ_{max} (nm)	$[M-H]^-$	MAIN FRAGMENT ESI- MSn [intensity (%)]	TENTATIVE IDENTIFICATION	QUANTIFICATION (mg/g of extract)
1	13.23	330	431	285(100)	Kaempherol-*O*-deoxy-hexoside	0.185±0.0003
2	13.68	330	431	285(100)	Kaempherol-*O*-deoxy-hexoside	0.185±0.0001
3	14.77	348	595	301(100)	Quercetin-*O*-pentosyl-hexoside	0.189±0.0005
4	15.73	347	595	301(100)	Quercetin-*O*-pentosyl-hexoside	0.186±0.0001
5	16.44	350	741	315(100)	Isorhamnetin-*O*-dipentosyl-hexoside	0.190±0.0002
6	18.04	340	609	315(100)	Isorhamnetin-*O*-pentosyl-hexoside	0.192±0.0004
					TFCNA	1.1273±0.0009
PEAK	**Tr (min)**	**λ_{max} (nm)**	**$[M+H]^+$**	**MAIN FRAGMENT ESI- MSn [intensity (%)]**	**TENTATIVE IDENTIFICATION**	**QUANTIFICATION (mg/g of extract)**
1’	17.69	535	433	287(100)	Cyanidin-*O*-deoxyhexoside	3.1±0.1
2’	20.66	535	417	271(100)	Pelargonidin-*O*-deoxyhexoside	1.37±0.05
					TFCA	4.4±0.2

Tr - retention time; TFCNA - total non-anthocyanin phenolic compounds; TFCA - total anthocyanin phenolic compounds. Calibration curves used: quercetin-3-O-glucoside (y = 34843x – 160173; R^2 = 0.9993); cyanidin-3-O-glucoside (y = 134578x – 3 x106; R^2 = 0.9986); pelargonidin-3-O-glucoside (y = 61493x - 628875; R^2 = 0.9957).

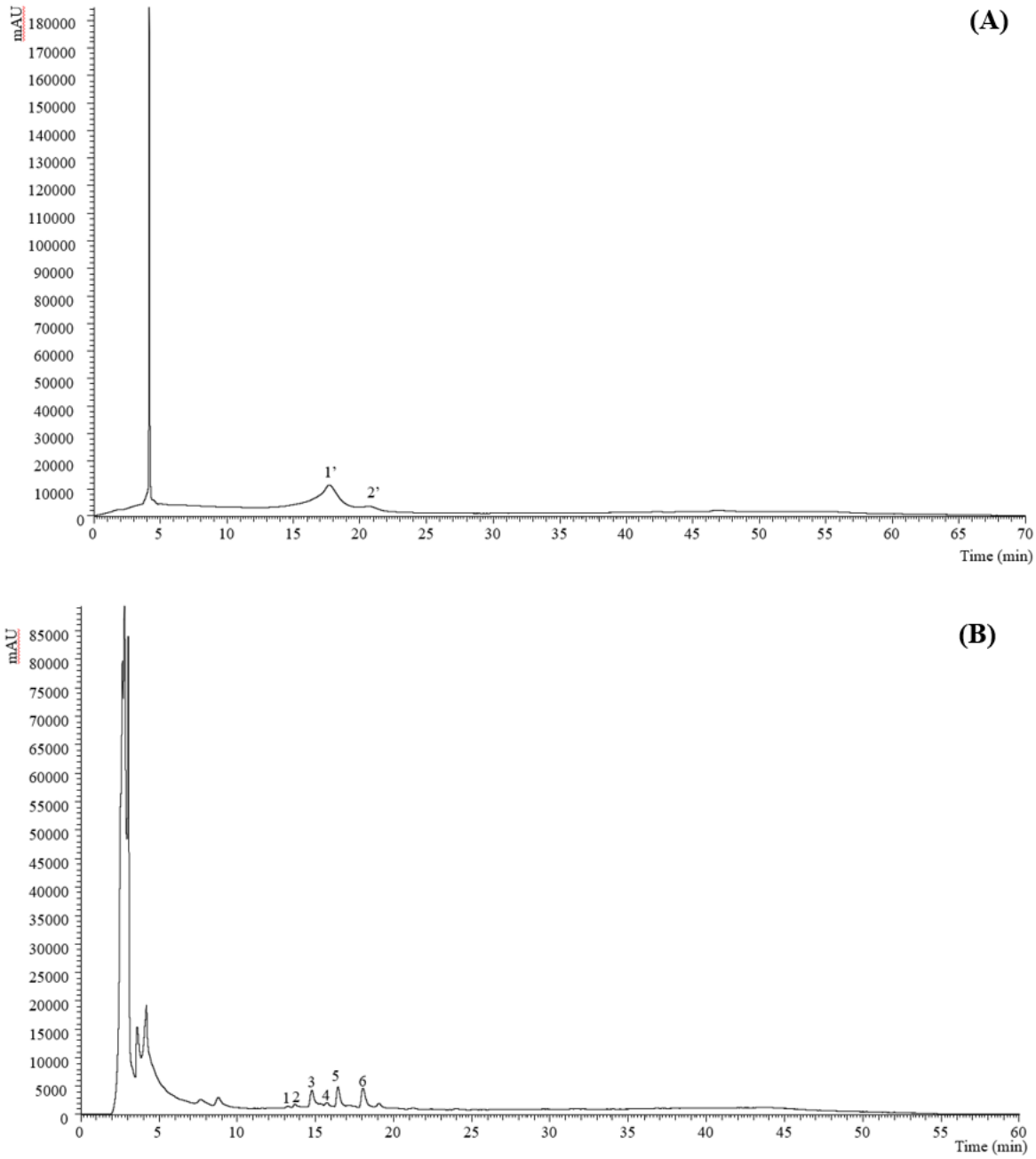


Figure 21. Profile of non-anthocyanin (A) and anthocyanin (B) phenolic compounds from the hydroethanolic extracts of *M. ermaginata* recorded at 280 nm (i) and 520 nm (ii). The peak number correspondence is shown in **Table 11**.

4.4. Evaluation of bioactive properties of the *Malpighia emarginata* DC. fruits, through *in vitro* tests

4.4.1. Antioxidant activity

The antioxidant activity of the *M. emarginata* extract obtained by the conventional method from its fruits was evaluated using two colorimetric *in vitro* assays (inhibition of lipid peroxidation – TBARS and oxidative hemolysis inhibition assay - OxHLIA). Results are showen in **Table 12**.

Considering the obtained values, the hydroethanolic extract of *M. emarginata* showed antioxidant potential in both performed assays. In this way in TBARS assay the EC_{50} value was 505 ± 20 µg/mL and for the OxHLIA assay the EC_{50} value was 231 ± 2 µg/mL.

The presence of antioxidant activity in the *M. emarginata* samples was expected due to the presence of compounds with bioactive capacity, such as them phenolic compounds.

Table 12. Antioxidant activity of the hydroethanolic extract of *Malpighia emarginata* DC fruits.

ANTIOXIDANT ACTIVITY	*M. emarginata*
TBARS (EC_{50} values, µg/mL)	505 ± 20
OxHLIA ($\Delta t = 60$ min) (EC_{50} values, µg/mL)	213 ± 2

EC_{50} values: Extract concentration corresponding to 50% of antioxidant activity.
Trolox (positive control) EC_{50} values: 5.4 ± 0.3 µg/mL (TBARS inhibition) and 22 µg/mL (OXHLIA).

Several studies were carried out to study the antioxidant potential, by *in vitro* assays, of different samples of *M. emarginata*. Rezende et al. (2017), studied the antioxidant activity from agro-industrial acerola residue. For this, *in vitro* assays such us DPPH radical and ABTS cation radical were applied. The results showed that pulp presented greater antioxidant activity, differing significantly with respect to the residue. This behavior may be related to ascorbic acid, since it is the only compound, most abundant in the pulp extract.

Other authors, Delva and Schneider (2013a), analyzed the antioxidant activity at different stages of fruit maturity and proved that when more mature were the fruits, the antioxidant activity was better. They have also carried out different extraction processes on fresh and freeze-dried fruit, obtaining better values in all cases in the ripe fruit.

Mezadri et al. (2008) also evaluated the antioxidant activity of *M. emarginata* fruits and derivates, such as crushed and frozen fruit, juice and frozen pulp. The antioxidant

capacity was evaluated using DPPH free radical, ABTS and ORAC methods and results revealed high antioxidant activity, but some differences in EC_{50} values, compared with the present study. These differences could be assigned to several treatments applied to the acerola fruits (crushed and squeezed) and, in some cases, maybe due to the month in which the fruits were harvested.

Other authors, such as Sancho et al. (2015) and Canuto et al. (2010), compared the antioxidant activity of different fruits, for example *M. emarginata*, açaí, tamarind, papaya, pineapple, mango, among other tropical fruits. In both studies, *M. emarginata* proved to be the fruit with the best antioxidant potential.

4.4.2. Antimicrobial activity

Table 13 show the results obtained for the antimicrobial activity of the hydroethanolic extract of *M. emarginata* fruis. The extract was tested against a panel of pathogenic bacteria (Gram-positive and Gram-negative bacteria) and fungi, chosen according to their influence in food sector and public health.

Regarding the antibacterial potential of the tested extract, the inhibitory and bactericidal capacity were evident in all the bacterial strains used. The MIC (minimal inhibitory concentration) values were 4.00 mg/mL for all strains, and de MBC (minimal bactericidal concentration) values range between 4.00 and 8.00 mg/mL. The most promising bactericidal activity was found to be against the strains *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimirium.* Even though higher extract concentrations were necessary in order to reach controls (E211 and E224) results. The best inhibitory capacity of *M. emarginata* extract was obtained for the *Staphylococcus aureus* strain, since MIC was reached in the same way than E211. Regarding the other strains, double or even higher concentrations of the hydroethanolic extract were required to completely inhibit them. As proposed by Settu and Manju (2017), this bioactive capacity may be related to the presence of isorhametin.

In terms of the antifungicidal potential, the obtained results showed remarkable inhibitory and fungicidal capacity of the extract against all the evaluated strains. The MIC values of *M. emarginata* ranged between 1.00 and 4.00 mg/mL, while for the minimal fungicidal concentration (MFC) values varied between 1.00 and 8.00 mg/mL. The best

activity was found against *Aspergillus fumigatus*, *Aspergillus versicolor* and *Trichoderma viride* strains, since MIC and MFC of the extract were the same or even lower than the controls (E211 and E224). Particullarly, MFC for *Trichoderma viride* strain of the extract was the half of the E211. Nevertheless, the inhibitory capacity of the extract was not as good as that of E211 and E224 against *Penicillium funiculosum* and *Penicillium aurantiogriseum* since higher amounts of extracts were needed for MIC and MFC.

Table 13. Antibacterial activity (MIC and MBC, mg/mL) and antifungal activity (MIC and MFC, mg/mL) of the hydroethanolic extracts obtained from *Malpighia emarginata* DC. fruit.

ANTIBACTERIAL ACTIVITY

		Staphylococcus aureus	*Bacillus cereus*	*Listeria monocytogenes*	*Escherichia coli*	*Salmonella typhimirium*	*Enterobacter cloacae*
M. emarginata	**MIC**	4.00	4.00	4.00	4.00	4.00	4.00
	MBC	8.00	8.00	4.00	4.00	4.00	8.00
E211	**MIC**	4.00	0.50	1.00	1.00	1.00	2.00
	MBC	4.00	0.50	2.00	2.00	2.00	4.00
E224	**MIC**	1.00	2.00	0.50	0.50	1.00	0.50
	MBC	1.00	4.00	1.00	1.00	1.00	0.50

ANTIFUNGAL ACTIVITY

		Aspergillus fumigatus	*Aspergillus niger*	*Aspergillus versicolor*	*Penicillium funiculosum*	*Penicillium aurantiogriseum*	*Trichoderma viride*
M. emarginata	**MIC**	1.00	2.00	1.00	2.00	4.00	1.00
	MFC	2.00	4.00	2.00	4.00	8.00	1.00
E211	**MIC**	1.00	1.00	2.00	1.00	2.00	1.00
	MFC	2.00	2.00	2.00	2.00	4.00	2.00
E224	**MIC**	1.00	1.00	1.00	0.50	1.00	0.50
	MFC	1.00	1.00	1.00	0.50	1.00	0.50

MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration; MFC: Minimal Fungicidal Concentration.
Sodium sulphite (E221) and potassium metabisulphite (E224) food additives were used as positive controls.

Motohashi et al. (2004) investigated the antimicrobial capacity of several extracts (hexane, ethyl acetate and 1-butanol) of *M. emarginata* and the results reported good antimicrobial properties of this fruit extracts against *Staphylococcus pidermidis* and relatively high anti-Gram-negative bacteria activity against *Escherichia coli* and *Psedomonas aeruginosa*.

In 2019, Barros et al. confirmed the antifungal activity of aqueous extract of *M. emarginata* leaves in clinically important species of *Candida* spp., showing significant growth inhibition of 50–90%, including death, mainly in *Candida tropicalis*, *Candida albicans*, *Candida krusei* and *Candida parapsilosis* strains.

The heterogeneity observed in the described studies can be partly justified by the different geographical location of the samples which, due to different environmental conditions, namely climate and soil type, lead to a different chemical composition. Other parameters that can affect the results are the solvents and extraction methods applied in each test, which have a great impact on the chemical composition of the extract and, consequently, on the bioactivities demonstrated.

4.4.3. Cytotoxicity, hepatoxicity and anti-inflammatory activity

The results obtained in the cytotoxicity and hepatotoxicity assays are presented in **Table 15**. Considering the values, it was evident that hydroethanolic extract (80:20 *v/v*), obtained from the fruit of *Malpigha emarginata* DC., manifested anti-proliferative capacity against the half tested tumour cell lines, namely CaCo and AGS, with values of 214 ± 5 µg/mL and 228 ± 7 µg/mL, respectively. In the other two tumour cell lines (NCI-H460 and MCF-7) no inhibition in cell growth was found, showing the anti-tumor incapacity of the hydroentolic extract of *M. emarginata*.

Regarding the evaluation of the hepatotoxicity performed in a primary non tumour cell culture (PLP2), the obtained value was 255 ± 20 µg/mL. The extract also showed an inhibitory capacity on cell proliferation; however, the concentrations obtained were higher compared to the tumour cell lines and the positive control, discarding the possibility of toxicity of this extract.

In relation to the anti-inflammatory potential, the result was 296 ± 2 µg/mL, showing anti-inflammatory activity.

Table 14. Cytotoxic and hepatotoxic activity of the hydroethanolic extract of *M. emarginata* fruits.

TUMOUR CELL LINES (values GI_{50}, µg/mL)	
MCF-7 (breast cancer)	>400
NCI-H460 (lung cancer)	>400
AGS (gastric cancer)	228±7
CaCo (colorectal cancer)	214±5
NON-TUMOUR CELL LINES (values GI_{50}, µg/mL)	
PLP2	255±20
ANTI-INFLAMMATORY (GI_{50} values; µg/mL)	
RAW264.7	296±2

PLP2: primary culture of pig liver cells; GI_{50} – concentration that inhibited 50% of cell growth.
Ellipticin (positive control): 1.02 ± 0.02 µg/mL (MCF-7), 1.01 ± 0.01 µg/mL (NCI-H460), 1.21 ± 0.02 µg/mL (CaCo), 1.23 ± 0.03 µg/mL (AGS); 1.4 ± 0.1 (PLP2).
Dexamethasone (positive control): 6.3 ± 0.4 µg/mL (RAW).
GI_{50} > 400 µg/mL- does not have activity.

Motohashi et al. (2004) investigated the cytotoxic activity in several extracts of *M. emarginata*, and they found high activity against human squamous cell carcinoma and human submandibular gland carcinomas. They have also shown that the cytotoxic activity of acerola extracts against two human tumor cells tended to decrease with the increase in the polarity of solvent used for extraction.

In 2018, Nagamine et al. carried out a treatment with water and tropical lemon juice extract powders from *M. emarginata* purees and leaves in rats. With the data obtained, they discovered that the components of the extract powders ameliorated the hepatic inflammatory response, decreased hepatocellular injury and improve liver function in rats subjected to acute D-galactosamine intoxication.

In 2015 Bhargava conducted a research project where he found that some leaf extracts and extracts of *M. emarginata* leaves and fruits have cyclooxygenase inhibitory effect for a number of genotypes. This author concluded that there is a high potential to be used as a natural source of non-steroidal anti-inflammatory drugs.

More recently, Castro (2017) also demonstrated the anti-inflammatory potential of *M. emarginata*. He worked with polysaccharides isolated from *M. emarginata* by-products, showing an anti-inflammatory effect in the naproxen-induced gastric ulcer model.

Albuquerque et al. in 2019, in their research determined the anti-inflammatory potential of water extracts of tropical fruits, such as passion fruit, orange, acerola and mango. Orange

water extract showed the highest anti-inflammatory effect, followed by mango, then acerola, and finally, passion fruit.

In this sense, the cytotoxic and anti-inflammatory potential of *M. emarginata* can be explained due to the presence of bioactive molecules in its composition, including phenolic and polysaccharide compounds.

4.5. Optimization of the extraction process to obtain a natural dye based on anthocyanins from the fruit of *Malpighia emarginata* DC.

The recovery of pigments from plant material has gained particular interest in recent years due to the current trend to develop natural colorants alternative to the artificial ones and to valorise and recycle agrofood by-products. However, the extraction of these molecules is affected by factors related to the intrinsic characteristics of the natural matrix and to the extraction method, such as temperature, processing time, type of solvent and solid/liquid ratio, among others. Therefore, several efforts have been made to develop more efficient and sustainable extraction processes, capable of improving extraction yield and selectivity for the target compounds (Pinela et al., 2019; Aluquerque et al., 2020; Rocha et al., 2020). However, the extrapolation of results obtained with different natural sources or using different extraction techniques can be a difficult task. In this study, the recovery of anthocyanins from *M. emarginata* bioresidues was optimized by RSM, considering the extraction time, temperature and ethanol concentration as independent variables.

4.5.1. Experimental data for extraction process optimization

The results of the 20 experimental runs of the CCRD design used to optimize the extraction of *M. emarginata* anthocyanins are shown in **Table 15**. The extraction yield ranged from 48% to 63% with runs 5 and 8, which combined medium-low (-1) time and temperature levels with medium-high (+1) solvent levels, or medium-high levels of the three independent variables, respectively. In turn, the levels of cyaninidin-*O*-deoxyhexoside (COD) and pelargonidin-*O*-deoxyhxoside (POD) ranged from 0.32–1.22 mg/g extract and 0.41–1.10 mg/g extract, respectively, and the total content varied from 0.73–2.32 mg/g extract. In both cases, the lowest contents were originated with run 5, the same that led to the lower extraction

yield, while the higher concentrations were reached with run 1, combining medium-low levels of the three variables.

Table 15. Experimental responses obtained under the extraction conditions defined by the CCRD design matrix for the extraction yield and anthocyanins content.

RUNS	EXPERIMENTAL DOMAIN			EXPERIMENTAL RESPONSES			
	t (min)	*T* (ºC)	*S* (%)	Yield (%)	COD (mg/g extract)	POD (mg/g extract)	Total (mg/g extract)
1	20	34	20	54.53	1.413	1.115	2.528
2	72	34	20	53.57	1.110	0.963	2.073
3	20	76	20	55.44	0.946	0.882	1.828
4	72	76	20	53.30	0.723	0.740	1.341
5	20	34	80	48.03	0.320	0.408	0.728
6	72	34	80	58.88	0.727	0.721	1.448
7	20	76	80	52.81	0.666	0.720	1.385
8	72	76	80	63.21	1.220	1.100	2.180
9	2	55	50	50.09	0.956	0.765	1.721
10	90	55	50	58.69	1.010	0.966	1.976
11	46	20	50	50.85	0.946	0.880	1.826
12	46	90	50	52.45	0.741	0.751	1.492
13	46	55	0	58.19	0.857	1.030	1.887
14	46	55	100	58.19	0.396	0.681	1.077
15	46	55	50	51.35	0.836	0.784	1.620
16	46	55	50	52.11	0.924	0.839	1.764
17	46	55	50	50.47	0.802	0.754	1.556
18	46	55	50	53.74	0.903	0.851	1.754
19	46	55	50	50.25	0.812	0.773	1.585
20	46	55	50	52.32	0.843	0.780	1.623

t: time; *T*: temperature; *S*: solvent; Yield: extraction yield; COD: cyaninidin-*O*-deoxyhexoside; POD: pelargonidin-*O*-deoxyhxoside; Total: total content of anthocyanins.

4.5.2. Models fitting and statistical verification

To construct the theoretical models, the experimental responses in **Table 15** were fitted to the second-order polynomial (**Equation 6**) using the Design-Expert software, but just the significant parameters ($p < 0.05$) were considered. The models, expressed in coded values, are presented **Equation 7** - **Equation 10**. The results of ANOVA and regression analyses are shown in **Table 16**.

$$Y_{Yield} = 51.7 + 2.39t + 0.91T + 3.04tS + 1.06TS + 0.96t^2 + 2.30S^2$$

Equation 7

$$Y_{COD} = 0.86 + 0.05t - 0.15S + 0.19tS + 0.21TS + 0.07t^2 - 0.07S^2$$

Equation 8

$$Y_{POD} = 0.83 + 0.05t - 0.10S + 0.12tS + 0.14TS$$

Equation 9

$$Y_{Total} = 1.66 + 0.07t - 0.25S + 0.31tS + 0.35TS + 0.07t^2 - 0.05S^2$$

Equation 10

In each model equation, the coefficients of the terms *t*, *T*, and *S* illustrate the effect of the independent variables time, temperature, and solvent, respectively, and their interactions. Since the expected effects on the response are denoted by the parametric values, the higher the parametric value, the more significant the weight of the respective variable, regardless of its sign. For interactions, synergistic effects are denoted by a positive sign, while an antagonistic interaction between variables is translated by a negative sign (Rocha et al., 2020). In each model **Equation 7** - **Equation 10**, the intercept corresponds to the overall average response of the 20 runs of the CCRD design in **Table 15**, being 51.7% for the extraction yield and 0.86, 0.86, and 1.66 mg/g extract for the dependent variables COD, POD, and Total, respectively.

All **Equation 7** - **Equation 10** presented a non-significant lack-of-fit ($p > 0.05$), which indicates that the theoretical models adequately describe the effects of the independent variables *t*, *T*, and *S* on the target responses (Iberahim et al., 2019). In all cases, the coefficients R^2 and R^2_{adj} were higher than 0.928 and 0.903, respectively (**Table 16**), indicating that the variability of each response can be explained by the independent variables involved in the extraction. In addition, values ≥ 22.6 were obtained for adequate precision, which is a measure of signal-to-noise ratio that compares the range of the predicted values at the design points to the average prediction error. High accuracy was also demonstrated by the low values of the coefficient of variance (CV). Thus, the developed theoretical models were statistically validated and used in the following steps to predict the optimal conditions for extraction of anthocyanins from *M. emarginata*.

Certain peculiarities regarding the overall effects of the independent variables on the extraction of *M. emarginata* anthocyanins can be inferred from the complexity of the model equations. Based on **Equation 6** and **Table 16**, it can be settled that the extraction yield was significantly affected by the three independent variables involved in the process. The extraction time was the most relevant process variable, effecting the extraction through positive linear and quadratic effects, and also strongly interacted with the solvent. The solvent ranked second and caused positive quadratic effects and also interacted with the temperature, which, in turn, caused linear effects on the response. On the other hand, **Equation 10** reflects the complexity of the extraction trend regarding the total content of anthocyanins, where it is interesting to note the negative linear and quadratic effects of the solvent and its positive interactions with the other two process variables. **Equation 8** and **Equation 9** showed extraction trends somewhat similar to those in model **Equation 10**, with parametric values more marked in **Equation 8**. Overall, these results support the use of RSM, since one-factor-at-a-time approaches do not assess interactive effects.

Table 16. Parametric values estimated with the polynomial **Equation 6** and statistical information of the model fitting procedure. Parametric superscripted 1, 2 and 3 stand for the independent variables time, temperature and solvent, respectively.

		YIELD	COD	POD	TOTAL
Intercept	b_0	51.7±0.4	0.86±0.02	0.83±0.01	1.66±0.03
Linear effect	b_1	2.4±0.3	0.05±0.02	0.05±0.01	0.07±0.02
	b_2	0.9±0.3	ns	ns	ns
	b_3	ns	-0.15±0.02	-0.10±0.01	-0.25±0.02
Quadratic effect	b_{11}	0.96±0.3	0.07±0.02	ns	0.07±0.02
	b_{22}	ns	ns	ns	ns
	b_{33}	2.3±0.3	-0.07±0.02	ns	-0.05±0.02
Interactive effect	b_{12}	ns	ns	ns	ns
	b_{13}	3.0±0.4	0.19±0.02	0.12±0.02	0.31±0.03
	b_{23}	1.1±0.4	0.21±0.02	0.14±0.02	0.35±0.03
Statistical information					
Model F-value		34.67	43.45	36.43	66.38
Lack of Fit		ns	ns	ns	ns
R^2		0.9529	0.9620	0.9286	0.9748
R^2_{adj}		0.9254	0.9399	0.9031	0.9601
Adequate Precision		22.60	28.20	26.58	36.81
CV (%)		1.92	7.11	6.07	4.67

Yield: extraction yield; COD: cyaninidin-*O*-deoxyhexoside; POD: pelargonidin-*O*-deoxyhxoside; Total: total content of anthocyanins; R^2: coefficient of determination; R^2_{ajd}: adjusted coefficient of determination; CV: coefficient of variation or relative standard deviation; ns: not significant.

4.5.3. Effect of the independent variables on the target responses and optimal extraction conditions

The response surface graphs built to visually illustrate the effect of the independent variables in the extraction of *M. emarginata* anthocyanins are presented in **Figure 22**. In each graph, the excluded variable was fixed at its individual optimal value shown in **Table 17**. The extraction yield (extract weight) was promoted by longer processing times at high temperatures and using higher ethanol concentrations. In fact, as evidenced by the parametric values of the interactive effects (**Table 16**), the increase in ethanol concentration induced a synergism on the extraction when combined with longer times (b_{13}) and, manly, with higher temperatures (b_{23}). The effect of the variables on the extraction yield is also shown in **Figure**

23, where 2D individual responses are illustrated. In each graph, the excluded variables were positioned at their optimal value.

Figure 23 also illustrate the contrary extraction trend observed for anthocyanins. The lower the variable ranges, the higher the recovery rate of anthocyanins. Moreover, the two anthocyanins presented similar response surfaces (**Figure 22**). In addition to low ethanol concentrations being more favourable for the recovery of these water-soluble vacuolar pigments, they seemed to be negatively affected by longer extraction times and higher temperatures, which may have caused their degradation. Therefore, it can be concluded that, although the 73.9 min processing at 62.7ºC with 85.8% ethanol maximizes the extraction yield to 63.9% (*w/w*) (**Table 17**), other compounds in addition to anthocyanins are being extracted. Interestingly, the conditions that favoured the extraction of anthocyanins were more sustainable, requiring a lower processing time (23.7 min), temperature (34.4ºC), and ethanol concentration (18.1%, *v/v*) to reach 2.55 mg/g extract. Thus, selectivity for anthocyanins can be achieved by applied this time-saving and eco-efficient extraction method. It should be noted that, during numerical optimization in Design-Expert software, the independent variables were set within the experimental range, while the response variables were set at maximum. In addition, equal "importance" of goals was also given to the variables.

For the industry interested in anthocyanin-rich extracts it is important to obtain a large amount of both extract weight and natural pigment through sustainable processes. Therefore, processing conditions that simultaneously maximize all responses were also determined (**Table 17**). Based on this second optimization step, 24.8 min extraction at 40.5ºC with 11.8% ethanol were found to be the optimal conditions to simultaneously maximize the extraction yield (57.1%, *w/w*) and anthocyanins content (2.54 mg/g extract) as much as possible. These conditions found for *M. emarginata* are comparable to those previously reported for the recovery of anthocyanins from jabuticaba (*Myrciaria jaboticaba* (Vell.) Berg.) epicarp (namely cyanidin-3-*O*-glucoside and delphinidin-3-*O*-glucoside; 21.8 min processing at 47.1 °C with 9.1% ethanol) (Albuquerque et al., 2020) and roselle (*Hibiscus sabdariffa* L.) calyces (cyanidin-3-*O*-sambubioside and delphinidin-3-*O*-sambubioside; 30 min processing at 30ºC with 0% ethanol) (Pinela et al., 2019), and more sustainable than those reported for the extraction of red raspberry (*Rubus idaeus* L.) anthocyanins (cyanidin-3-*O*-sophoroside and

cyanidin-3-*O*-glucoside; 75.7 min processing at 38.1ºC with 21.4% ethanol) (Rocha et al., 2020).

Table 17. Optimal conditions that maximize the extraction of anthocyanins from *M. emarginata*.

	OPTIMAL PROCESSING CONDITIONS			**RESPONSE OPTIMUM**
	t (min)	*T* (ºC)	*S* (%)	
Individual conditions for each response variable				
Extraction yield	73.9	62.7	85.8	63.9±0.8% (*w/w*)
Cyaninidin-*O*-deoxyhexoside	20.0	33.5	21.3	1.38±0.04 mg/g extract
Pelargonidin-*O*-deoxyhxoside	36.3	42.9	0.0	1.19±0.03 mg/g extract
Total anthocyanins	23.7	34.4	18.1	2.55±0.06 mg/g extract
Global conditions considering all response variables				
Extraction yield	24.8	40.5	11.8	57.1±0.8% (*w/w*)
Cyaninidin-*O*-deoxyhexoside				1.35±0.05 mg/g extract
Pelargonidin-*O*-deoxyhxoside				1.16±0.04 mg/g extract
Total anthocyanins				2.54±0.06 mg/g extract

t: time; *T*: temperature; *S*: solvent.

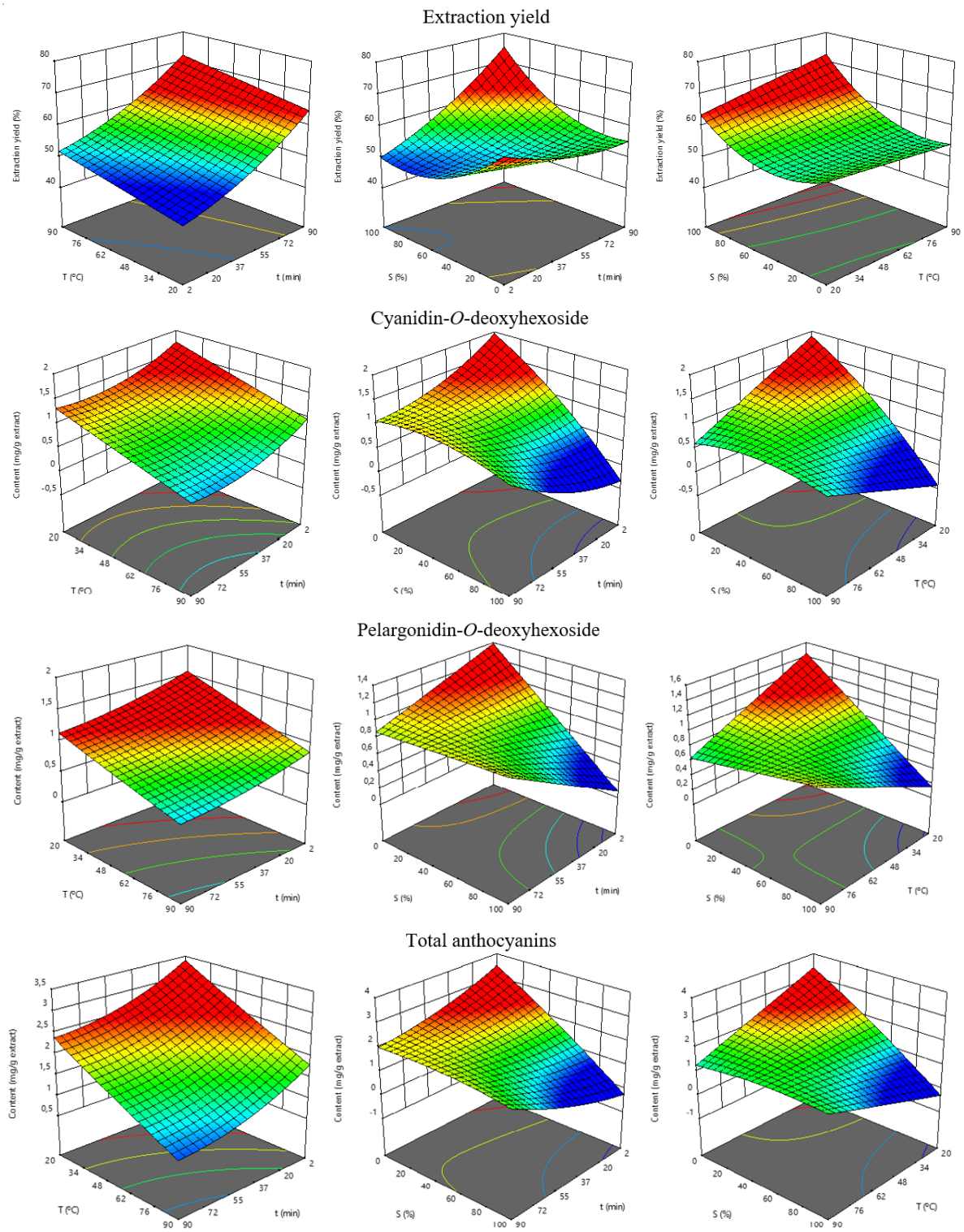


Figure 22. Response surface graphs for the combined effects of the independent variables time (t), temperature (T) and solvent (S) on the extraction yield and anthocyanins content obtained from *M. emarginata* bioresidues. In each graph, the excluded variable was fixed at its optimal value.

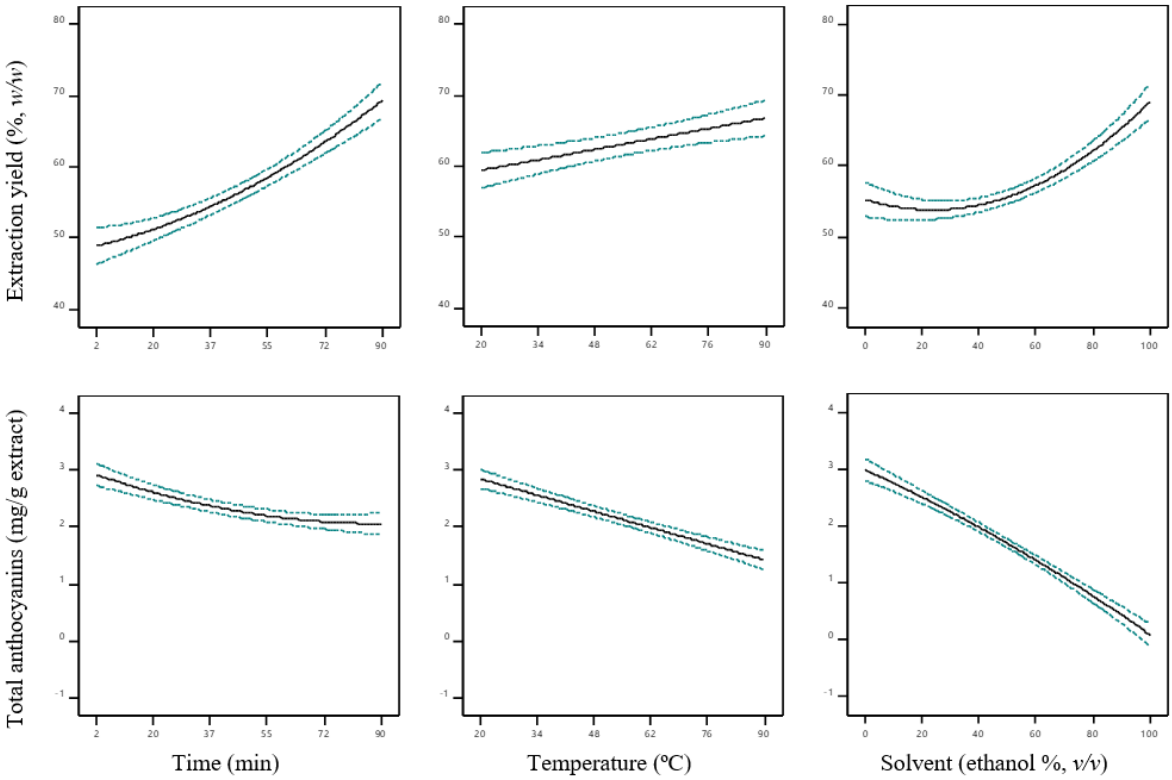


Figure 23. 2D response graphs for the effects of the independent variables on the extraction yield and total anthocyanins obtained from *M. emarginata* bioresidues. In each graph, the excluded variables were fixed at their optimal value.

Conclusions and Perspectives

5. Conclusions and Perspectives

The increase in the consumption of healthy food and the large amount of organic waste generated by the industry, lead the scientific community and industrial sector to establish solutions that promote the use and valorization of these residues.

In this sense, the nutritional and chemical characterization of *M. emarginata* bioresidues, as well as the evaluation of bioactive potential, using several in vitro assays were done. Furthermore, a study of extraction optimization was performed, in order to obtain a natural colourant pigment rich in anthocyanins.

The nutritional composition revealed that *M. emarginata* is a good source of energy, since carbohydrates were detected as the main macronutrient. Otherwise, the chemical profile showed fructose as the predominant sugar, while, among all organic acids detected, malic acid presented the highest concentration. Regarding the fatty acids' composition, fourteen different molecules were detected and the oleic acid was the main one.

In terms of phenolic compounds, anthocyanins presented 79.6% in relation to the total content of phenolic compounds, with cyanidin-*O*-deoxyhexoside being the predominant compound, followed by the pelargonidin-*O*-deoxyhexoside. Regarding the non-anthocyanin compounds, six types were found. The concentrations obtained for these were similar in all of them, however, isorhamnetin-*O*-pentosyl-hexoside was the majority.

The bioactive potential of the *M. emarginata* hydroethanolic extract revealed that the antioxidant, antimicrobial and cytotoxic action maybe due to the presence of compounds with bioactive capacity, namely phenolic compounds.

In addition, in order to optimize the extraction of anthocyanins from this fruit, to obtain a colouring ingredient rich in anthocyanins, a study was carried out using the response surface method (RSM) in order to maximize the extractability of all the individual compounds, as well as the total anthocyanin content. The optimal conditions established were: t= 24.8 min, T= 40.5 ºC, S= 11.8 % (ethanol) conducting to an extraction yield of 57.1%, with a total anthocyanins' content of 2.54 mg/g extract.

Based on the obtained results during this work, it can be concluded that the fruits of *M. emarginata* could have a potential value as functional food ingredients, due to their nutritional and chemical composition, as well as, the antioxidant and antibacterial activity action.

Moreover, considering its composition rich in anthocyanin compounds, it could be used as an alternative natural matrix to obtain a natural colouring ingredient not interfering in the food chain, since it is about the use of fruits that do not have the necessary characteristics for commercialization.

All these results demonstrate that biowaste discarded by the food industry, as is the case of *M. emarginata* fruits, can have a beneficial purpose for humans and the environment, as well as the possibility of having a high added value when processed and transformed.

Regarding the food industry, the valorization of biowaste has gained prominence as an alternative option to eliminate its accumulation, reduce the cost of its degradation and promote the development of the circular economy.

Furthermore, this research has increased the possibilities for different technologies to modify traditional foods through the addition of biologically active compounds in food matrices. Evidencing its benefits on human health, mainly the reduction of the risk of diseases.

In order to give continuity to this experimental work, as future perspectives, it would be interesting to study the stability of the extract and the evaluation of different techniques for its incorporation in several food products. Furthermore, the positive results obtained in terms of antimicrobial activity demonstrated the possibility of applying this extract not only as a natural dye, but also as a preservative ingredient, inhibiting the development of microorganisms.

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